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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(21) International Application Number:</b> PCT/US97/24051<br><b>(22) International Filing Date:</b> 23 December 1997 (23.12.97)<br><b>(30) Priority Data:</b><br>08/774,104 24 December 1996 (24.12.96) US<br><b>(71) Applicant (for all designated States except US):</b> THE JOHNS HOPKINS UNIVERSITY [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).<br><b>(72) Inventor; and</b><br><b>(75) Inventor/Applicant (for US only):</b> CHATTERJEE, Subroto [US/US]; 6098 Sebring Drive, Columbia, MD 20144 (US).<br><b>(74) Agents:</b> CORLESS, Peter, F. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109 (US). |           | <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i> |
| <b>(54) Title:</b> RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME<br><br><b>(57) Abstract</b><br><br>Isolated nucleic acids are provided that encode human neutral sphingomyelinase (N-SMase) and N-SMase fragments and derivatives capable of hybridizing to such N-SMase-encoding nucleic acids. The invention also includes isolated recombinant human neutral sphingomyelinase (N-SMase) and N-SMase fragments and derivatives are also provided. Novel assays are also provided to identify compounds useful in the diagnosis or treatment of human neutral sphingomyelinase related disorders.  |           |   |

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## RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME

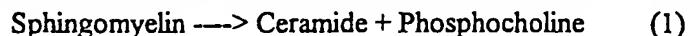
## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The present invention relates to human neutral sphingomyelinases (N-SMases), including recombinant N-SMases and fragments and derivatives thereof and isolated  
5 nucleic acids encoding N-SMases and fragments and derivatives. In preferred aspects, assays for identifying compounds that can modulate N-SMase related activity are provided, particularly assays to identify a pharmacological agent useful in the diagnosis or treatment of disorders associated with human neutral sphingomyelinases.

## 2. Background

10 Sphingomyelinases type-C (E.C. 3.1.4.12) are a group of phospholipases that catalyze the hydrolytic cleavage of sphingomyelin via the following reaction (1).



Native N-SMase purified from human urine and cultured human kidney proximal tubular cell membranes has an apparent molecular weight of 92 kDa, neutral  
15 pH optima, is heat unstable and is localized on the surface of various cells. S. Chatterjee, *Adv. Lipid Res.*, 26:25-48 (1993); S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989); and S. Chatterjee et al., *Methods in Enzymology, Phospholipase*, 197:540-547 (1991). N-SMase action has been shown to mediate signal transduction of vitamin D<sub>3</sub>, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon-gamma  
20 and nerve growth factor (Y. Hannun, *J. Biol. Chem.*, 269:3,125-3,128 (1994); S. Chatterjee, *J. Biol. Chem.*, 268:3,401-3,406 (1993); and S. Chatterjee, *J. Biol. Chem.*, 269:879-882 (1994)) leading to cell differentiation in human leukemic (HL-60) cells and insulin signaling (P. Peraldi et al., *J. Biol. Chem.*, 271:13018-13022 (1996)).

In addition to the biological roles of sphingomyelin and ceramide in signal  
25 transduction pathways involving cell regulation, recent evidence suggests that sphingomyelinases may be involved in the mobilization of cell surface cholesterol, in cholesterol ester synthesis, and in induction of low density lipoprotein (LDL) receptor activity. See S. Chatterjee, *Advances in Lipid Research*, 26:25-48 (1993). Recent evidence also supports a possible role of ceramide (a product of N-SMase activity) in

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programmed cell death and/or "apoptosis" and activation of the gene for nuclear factor (NF)-kB. See A. Alessenko and S. Chatterjee, *Mol. Cell. Biochem.*, 143:169-174 (1995). Sphingomyelinases are also believed to serve as a signal for various exogenous effectors such as antibiotics, drugs, and growth factors, which influence the normal physiology of cells.

A number of specific disorders have been associated with N-SMase. For example, N-SMase has been reported to be associated with insulin resistant diabetes and obesity. See Speigel et al., *J. Biol. Chem.*, 1996. N-SMase is also associated with malaria. The development of the malaria parasite plasmodium requires N-SMase. See Lauer et al., *Proc. Nat. Acad. Sci. (USA)*, 1995. N-SMase also is involved in liver cell proliferation. See Alessenko, Chatterjee, *Mol. Cell Biochem.*, 143:169-174 (1995).

Thus, methods for identifying agents which can modulate N-SMase activity would be highly useful. Moreover, methods for identifying pharmacological agents of interest by automated, high throughput drug screening have become increasingly relied upon in a variety of pharmaceutical and biotechnology drug development programs. Unfortunately, however, requisite reagents for such high throughput screening assays to identify agents potentially useful in treatment of N-SMase associated disorders are not readily available. For example, current methods for procuring N-SMase include isolation of the protein from substantial quantities of urine. See, for example, S. Chatterjee, *J. Biol. Chem.*, 264(21):12554 (1989).

It thus would be desirable to have a convenient source of N-SMases. It also would be desirable to have agents that can modulate N-SMase activity. It would be further desirable to have effective assays for identifying compounds that have the potential to modulate N-SMase activity or to diagnose or treat disorders relating to N-SMase.

#### SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acids that encode human neutral sphingomyelinases (N-SMase) and N-SMase fragments and derivatives capable of hybridizing to such N-SMase-encoding nucleic acids. cDNA (SEQ ID NO:1) encoding human N-SMase has been isolated and expressed to provide recombinant N-SMase having an apparent molecular weight of 44 kDa.

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The invention further provides isolated recombinant human neutral sphingomyelinase (N-SMase) and N-SMase fragments and derivatives.

The invention also provides novel assays for identifying compounds useful in the diagnosis or treatment of human neutral sphingomyelinase related disorders.

- 5 Preferred compounds identified through assays of the invention can modulate, particularly inhibit, human neutral sphingomyelinase activity.

A variety of such assays are provided including, e.g., cleavage assays, direct binding assays, as well as assays that identify a particular domain function. A preferred assay of the invention comprises providing 1) an isolated human neutral  
10 sphingomyelinase or a fragment or derivative thereof, 2) a human neutral sphingomyelinase cleavage target such as sphingomyelin, and 3) a candidate pharmacological agent potentially useful in the diagnosis or treatment of disease associated with human neutral sphingomyelinase, which agents 1), 2) and 3) are typically assayed in admixture. Those agents are suitably treated under conditions  
15 whereby, but for the presence of the candidate pharmacological agent, the N-SMase or fragment or derivative thereof selectively cleaves the cleavage target to yield a cleavage product such as ceramide. The agents are then analyzed for the presence of the cleavage product, wherein the absence or reduced concentration (e.g. relative to control, i.e. same mixture of agents 1) and 2) but without the candidate agent 3)) of  
20 the cleavage product indicates that the candidate pharmacological agent is capable of modulating N-SMase activity, particularly inhibition of sphingomyelin cleavage activity.

Agents identified through assays of the invention will have potential for use in a number of therapeutic applications, especially to modulate, particularly inhibit,  
25 expression or activity of human neutral sphingomyelinase in particular cells. Specific disorders that potentially could be treated by administration of pharmacological agents identified through assays of the invention include inflammatory disorders such as arthritis and osteoarthritis, treatment of obesity and diabetes, treatment of malignancies, and treatment of HIV. Identified agents also may be useful for treatment  
30 of cirrhosis of the liver and other liver diseases, to increase human plasma low density

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lipoproteins receptors (to thereby reduce excessive cholesterol levels of a subject), and for treatment of atherosclerosis.

Identified agents also may be useful for *in vitro* fertilization applications, particularly to improve viability and/or effective lifetime of sperm or seminal fluid samples during storage. Identified agents also may be useful to treat or inhibit undesired vascular restenosis e.g. subsequent to arterial plaque removal. Identified agents also may be useful in the treatment of central nervous system disorders such as treatment of depression, schizophrenia and Alzheimer's disease, and treatment or prevention or inhibition of neurodegeneration. Identified agents also may be useful to prevent transmission of malaria by application of the agent to areas frequented by malaria carriers to thereby prevent development of the parasite plasmodium.

The invention further provides methods to modulate expression or activity of N-SMase in particular cells through administering to a patient in need thereof a therapeutically effective amount of human neutral sphingomyelinase or N-SMase fragment or derivative thereof or a nucleic acid encoding same. Preferably, an N-SMase fragment or derivative or corresponding nucleic acid is administered that contains only selected domains to thereby modulate N-SMase activity as desired and without effects associated with the deleted or otherwise altered domain(s). For instance, as discussed in more detail below, it will be generally preferred the TSLKVPA domain of N-SMase or corresponding nucleic acid sequence will not be present in functional form in administered peptides or nucleic acids.

Such therapeutic methods can be employed to treat subjects susceptible to (i.e. prophylactic treatment) or suffering from N-SMase related disorders including e.g. inflammatory disorders such as arthritis and osteoarthritis, Crohn's disease, treatment of obesity and diabetes, treatment of malignancies, particularly cancers including susceptible solid tumors, treatment of HIV, treatment of renal failure, and to prevent or inhibit transmission of malaria. N-SMase or fragments or derivatives thereof, and nucleic acids encoding same of the invention, also can be used for treatment of cirrhosis of the liver and other liver diseases, and to increase human plasma low density lipoproteins (LDL) receptors and to thereby reduce excessive cholesterol levels. N-



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SMase or fragments or derivatives thereof, and nucleic acids encoding same, also may be used for treatment of atherosclerosis.

5 N-SMase or fragments or derivatives thereof, and nucleic acids encoding same, also may be used for *in vitro* fertilization applications, particularly to improve viability and/or effective lifetime of sperm or seminal fluid samples during storage. In this aspect, the invention provides sperm or seminal fluid in combination with an N-SMase fragment or derivative to thereby provide enhanced viability or lifetime of samples during the storage period. The sperm or seminal fluid sample may be human, or of other mammal such as horse, cattle or other livestock.

10 N-SMase or fragments or derivatives, and nucleic acids encoding same, can be further employed to treat or inhibit undesired vascular restenosis e.g. subsequent to arterial plaque removal, and the treatment of central nervous system disorders such as treatment of depression, schizophrenia and Alzheimer's disease, and treatment or prevention or inhibition of neurodegeneration.

15 N-SMase or fragments or derivatives, and nucleic acids encoding same, particularly fragment or derivatives and nucleic acids that inhibit N-SMase activity, also may be administered to treat a patient, particularly a human, suffering from or susceptible to cardiac disease where LV dysfunction occurs, including a patient, particularly a human, suffering from or susceptible to heart failure.

20 Other aspects of the invention are disclosed *infra*.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nucleotide sequence (SEQ ID NO:1) of isolated cDNA encoding human N-SMase.

25 Figure 2 shows the deduced amino acid sequence (SEQ ID NO:2) of human N-SMase. ♦ N-glycosylation site, • tyrosine kinase phosphorylation site, ♣ protein kinase phosphorylation sites, ♥ casein kinase II phosphorylation sites, ▽ cyclic AMP and cyclic GMP dependent protein phosphorylation sites, underline: myristoylation sites, \* stop codon.

Figure 3 shows the hydropathy plot analysis of N-SMase.

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Figures 4A and 4B show gel electrophoretic analysis of recombinant N-SMase expressed in *E. coli*. Figure 4A shows a Comassie blue staining and Figure 4B shows Western immunoblot analysis.

Figure 5 shows transient expression of recombinant N-SMase in Cos-7 cells.

5        Figure 6A and 6B show Northern blot analyses of N-SMase. Figure 6A shows tissue distribution of N-SMase. Figure 6B shows transcript size of human kidney N-SMase.

Figure 7 shows N-SMase activity in aortic smooth muscle cells transiently transfected with cDNA for N-SMase.

10        Figures 8A, 8B and 8C show chromatin condensation in transiently transfected aortic smooth muscle cells. Figure 8A shows control cells. Figure 8B shows PVS-SPOT. Figure 8C shows PHH1.

#### DETAILED DESCRIPTION OF THE INVENTION

We have now isolated cDNA encoding a human N-SMase. This cDNA is represented by SEQ ID NO:1 (Figure 1) and encodes a protein that when expressed in  
15        *E. coli* cells has an apparent molecular weight of 44 kDa as determined by polyacrylamide gel electrophoresis using sodium laurylsarocine. That recombinant protein is bound by an antibody against the 92 kDa native N-SMase. Protein was also expressed in Cos-7 cells. The isolated and purified recombinant N-SMase has been  
20        shown to have N-SMase activity. See, for instance, the results disclosed in Example 1 which follows.

As discussed above, the ability to regulate N-SMase activity in a particular environment is very important. Too high a level of N-SMase or conversely too low a level of N-SMase can result in undesired effects. For example, excessively high N-  
25        SMase levels can result in apoptosis, while excessively low levels of N-SMase can result in lack of cell proliferation or lack of LDL receptors. Similarly, N-SMase expression in one cell type can be desirable in leading to more efficient cholesterol processing, whereas its expression in another cell type can be undesirable.

As discussed above, the invention provides methods to modulate, including  
30        inhibition of, expression or activity of N-SMase in particular cells. For example, one can use a N-SMase nucleic acid segment operably linked to a N-SMase promoter to

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selectively direct it to desired cells. As another example, one can administer a N-SMase protein or fragment or derivative to modulate N-SMase activity.

The invention further provides isolated N-SMase having an amino acid sequence represented by SEQ ID NO:2 (Figure 2), as well as fragments or derivatives thereof.

The term "fragment" or "derivative" when referring to an N-SMase protein means proteins or polypeptides which retain essentially the same biological function or activity as the protein of SEQ ID NO:2. For example, the N-SMase fragments or derivatives of the present invention maintain at least about 50% of the activity of the protein of SEQ ID NO:2, preferably at least 75%, more preferably at least about 95% of the activity of the protein of SEQ ID NO:2, as determined e.g. by a standard activity gel assay such as the assay disclosed in Example 1, part 6, which follows and includes measuring activity of the N-SMase peptide using [<sup>14</sup>C]-sphingomyelin.

Fragments or derivatives as the term is used herein can include competitors of the native N-SMase with respect to a particular N-SMase domain activity. However, the fragment or derivative shows an overall similarity to N-SMase in other areas as explained herein.

An N-SMase fragment or derivative of the invention may be (i) a peptide in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) a peptide in which one or more of the amino acid residues includes a substituent group, or (iii) a peptide in which the mature protein is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol). Thus, an N-SMase fragment or derivative includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The protein fragments and derivatives of the invention are of a sufficient length to uniquely identify a region of N-SMase. N-SMase fragments and derivatives thus preferably comprise at least 8 amino acids, usually at least about 12 amino acids, more usually at least about 15 amino acids, still more typically at least about 30 amino acids,

even more typically at least about 50 or 70 amino acids. Preferred N-SMase fragments or derivatives of the invention include those that have at least about 70 percent homology (sequence identity) to the protein of SEQ ID NO:2, more preferably about 80 percent or more homology to the protein of SEQ ID NO:2, still more preferably about 85 to 90 percent or more homology to the protein of SEQ ID NO:2.

N-SMase and fragments and derivatives thereof of the invention are "isolated", meaning the protein or peptide constitutes at least about 70%, preferably at least about 85%, more preferably at least about 90% and still more preferably at least about 95% by weight of the total protein in a given sample. A protein or peptide of the invention preferably is also at least 70% free of immunoglobulin contaminants, more preferably at least 85% free, still more preferably at least 90% free and even more preferably at least 95% free of immunoglobulin contaminants. The N-SMase fragments and derivatives may be present in a free state or bound to other components, e.g. blocking groups to chemically insulate reactive groups (e.g. amines, carboxyls, etc.) of the peptide, or fusion peptides or polypeptides (i.e. the peptide may be present as a portion of a larger polypeptide).

As discussed above, N-SMase nucleic acid fragments and derivatives are also provided. Those fragments and derivatives are of a length sufficient to bind to the sequence of SEQ ID NO:1 under the following moderately stringent conditions (referred to herein as "normal stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.8M saline/0.08M sodium citrate (SSC) buffer at a temperature of 37°C and remaining bound when subject to washing once with that SSC buffer at 37°C.

Preferred N-SMase nucleic acid fragments and derivatives of the invention will bind to the sequence of SEQ ID NO:1 under the following highly stringent conditions (referred to herein as "high stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.9M saline/0.09M sodium citrate (SSC) buffer at a temperature of 42°C and remaining bound when subject to washing twice with that SSC buffer at 42°C.

These nucleic acid fragments and derivatives preferably should comprise at least 20 base pairs, more preferably at least about 50 base pairs, and still more

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preferably a nucleic acid fragment or derivative of the invention comprises at least about 100, 200, 300, 400, 500 or 800 base pairs. In some preferred embodiments, the nucleic acid fragment or derivative is bound to some moiety which permits ready identification such as a radionucleotide, fluorescent or other chemical identifier.

5 Preferred nucleic acid fragments or derivatives of the invention include those that have at least about 70% homology (sequence identity) to the SEQ ID NO: 1, more preferably at least about 80% homology to SEQ ID NO: 1, still more preferably at least about 85%, 90%, 95% or 98% homology to SEQ ID NO: 1.

N-SMase will have a number of functional domains, e.g., a TNF- $\alpha$  55kDa  
10 receptor/Fas Apo(o)-1 domain, the sterol regulator element binding protein (SREBP) domain, etc. N-SMase of Figure 2 also has a domain (TSLKVPA, residues 258-264 of Figure 2; SEQ ID NO:3) homologous to the Staphyococcal enterotoxin-B peptide domain (RSITVRV; SEQ ID NO:4), which domain has been reported to elicit toxic effects in human kidney cells. Accordingly, preferred N-SMase fragments and  
15 derivatives do not include that TSLKVPA domain in functional form. Other N-SMase domains can be readily identified by standard techniques such as deletion analysis.

In a similar manner, one can readily identify a deletion, addition or substitution that will inactivate a particular domain, e.g. by simply testing a fragment or derivative with altered domain to determine if the fragment or derivative exhibits activity  
20 associated with the altered domain. Any of a variety of tests can be employed, such as e.g. the *in vitro* tests of Example 1 which follows.

Thus, an N-SMase protein or nucleic acid fragment or derivative can be employed that contains only specific domains and can be administered to a subject such as a mammal to modulate N-SMase activity in targeted cells as desired.

25 Preferred N-SMase protein and nucleic acid fragments and derivatives include at least one functional domain region, e.g. the TNF- $\alpha$  binding domain, the SREBP domain or the sphingomyelin cleavage domain. Particularly preferred fragments and derivatives comprise one or more conserved peptides or corresponding nucleic acid sequences of at least one functional domain region.

30 Several apparent isoforms of human N-SMase exist that can be distinguished based on physical-chemical criteria, including electrophoretic migration rates, reactions

against antibody of N-SMase and reactions to metals including copper, lithium and magnesium. The N-SMase and fragments and derivatives thereof of the present invention, and nucleic acids encoding same, include such isoforms.

Isolated N-SMase and peptide fragments or derivatives of the invention are preferably produced by recombinant methods. See the procedures disclosed in Example 1 which follows. A wide variety of molecular and biochemical methods are available for generating and expressing the N-SMase of the present invention; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Seidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art. For example, N-SMase or fragments thereof may be obtained by chemical synthesis, expression in bacteria such as *E. coli* and eukaryotes such as yeast, baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of the N-SMase or fragment. The use of mammalian-based expression systems, particularly human, is particularly preferred where the peptide is to be used therapeutically.

Nucleic acids encoding the novel N-SMase of the present invention and fragments and derivatives thereof may be part of N-SMase expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals for functional studies (e.g. the efficacy of candidate drugs for disease associated with expression of a N-SMase), etc. Nucleic acids encoding N-SMase containing proteins are isolated from eukaryotic cells, preferably human cells, by screening cDNA libraries with probes or PCR primers derived from the disclosed N-SMase cDNAs.

The nucleic acids of the present invention are isolated, meaning the nucleic acids comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome and usually constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least

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about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

The nucleic acids of the present invention find a wide variety of applications including: use as translatable transcripts, hybridization probes, PCR primers, therapeutic nucleic acids, etc.; use in detecting the presence of N-SMase genes and gene transcripts; use in detecting or amplifying nucleic acids encoding additional N-SMase homologs and structural analogs; and use in gene therapy applications.

For example, N-SMase nucleic acids can be used to modulate cellular expression or intracellular concentration or availability of active N-SMase. Thus, for example, N-SMase has been shown to be involved in liver cell proliferation (Alessenko and Chatterjee, *Mol. Cell. Biochem.* 143:119-174, (1995)), and thus N-SMase nucleic acids may be used to treat liver diseases such as cirrhosis.

To inhibit N-SMase activity, nucleic acid encoding a competitor or an antagonist can be administered to a subject. One preferred embodiment employs nucleic acid encoding an N-SMase derivative that acts as a competitor or an antagonist. For example, dependent upon the N-SMase activity desired to be inhibited, the N-SMase domain responsible for that activity can be appropriately altered (e.g. deleted or mutated), whereby the protein will still display the desired activity, but will not exhibit the undesired activity. Moreover, the altered protein can compete with the native N-SMase to thereby inhibit the undesired activity.

Further, to reduce N-SMase activity, nucleic acids capable of inhibiting translation of N-SMase also may be administered. These nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed relevant N-SMase fragment-encoding nucleic acid. Antisense modulation of the expression of a given N-SMase fragment containing protein may employ N-SMase fragment antisense nucleic acids operably linked to gene regulatory sequences. Cells are transfected with a vector comprising an N-SMase fragment sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous N-SMase fragment containing protein encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration.

Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a given N-SMase fragment containing protein may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in expression of the N-SMase.

5           The N-SMase nucleic acids are introduced into the target cell by any method which will result in the uptake and expression of the nucleic acid by the target cells. These can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, catheters, etc. Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid  
10 binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

15           Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox vectors, herpes virus vectors such as a herpes simplex I virus (HSV) vector [A.I. Geller et al., *J. Neurochem*, 64:487 (1995); F. Lim et al., in *DNA Cloning:*  
20 *Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); A.I. Geller et al., *Proc Natl. Acad. Sci.*: U.S.A.:90 7603 (1993); A.I. Geller et al., *Proc Natl. Acad. Sci USA*: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., *Nat. Genet.*, 3:219 (1993); Yang et al., *J. Virol.*, 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al.,  
25 *Nat. Genet.*, 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a  
30 shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will



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depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA,  $\text{Ca}_3(\text{PO}_4)_2$  precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, and

5 viral vectors.

The vector can be employed to target essentially any desired target cell, such as a glioma. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Additionally, the particles can be delivered by intracerebroventricular (icv) infusion using a minipump infusion system, such as a

10 SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell (Bobo et al., *Proc. Natl. Acad. Sci. USA*, 91:2076-2080 (1994); Morrison et al., *Am. J. Physiol.*, 266: 292-305 (1994)). Other methods that can be used include catheters, intravenous, parenteral,

15 intraperitoneal and subcutaneous injection, and oral or other known routes of administration.

The invention provides efficient screening methods to identify pharmacological agents or lead compounds for agents which modulate, e.g. interfere with or increase an N-SMase activity. The methods are amenable to automated, cost-effective high

20 throughput drug screening and have immediate application in a broad range of pharmaceutical drug development programs.

A wide variety of assays are provided including, e.g., cleavage assays, direct binding assays as well as assays to identify a particular domain function.

A preferred assay mixture of the invention comprises at least a portion of the

25 N-SMase capable of cleaving a N-SMase cleavage target, e.g. sphingomyelin. An assay mixture of the invention also comprises a candidate pharmacological agent. Generally a plurality of assay mixtures are run in parallel with different candidate agent concentrations to obtain a differential response to the various concentrations. Typically, one of these assay mixtures serves as a negative control, i.e. at zero

30 concentration or below the limits of assay detection. Candidate agents encompass numerous chemical classes, though typically they are organic compounds and

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preferably small organic compounds. Small organic compounds suitably may have e.g. a molecular weight of more than about 50 yet less than about 2,500. Candidate agents may comprise functional chemical groups that interact with proteins and/or DNA.

Candidate agents are obtained from a wide variety of sources including libraries  
5 of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced.

10 Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc.

A variety of other reagents may also be included in the mixture. These include  
15 reagents such as salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

20 The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the N-SMase or fragment or derivative thereof cleaves the cleavage target. The mixture components can be added in any order that provides for the requisite bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4° and 40°C, more  
25 commonly between 15° and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high throughput screening, and are typically between 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

After incubation, the presence or absence of the cleavage product is detected  
30 by any convenient way. For cell-free type assays, the cleavage target may be bound to a solid substrate and the cleavage product labelled, e.g., radiolabelled. A separation

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step can be used to separate the bound target from unbound cleavage product. The separation step may be accomplished in a variety of ways known in the art. The solid substrate may be made of a wide variety of materials and in a wide variety of shapes, e.g. microtiter plate, microbead, dipstick, resin particle, etc. The substrate is chosen to maximize signal to noise ratios, to minimize background binding, to facilitate washing and to minimize cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead (e.g. beads with iron cores may be readily isolated and washed using magnets), particle, chromatographic column or filter with a wash solution or solvent. Typically, the separation step will include an extended rinse or wash or a plurality of rinses or washes. For example, where the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific binding such as salts, buffer, detergent, nonspecific protein, etc. may exploit a polypeptide specific binding reagent such as an antibody or receptor specific to a ligand of the polypeptide.

As mentioned, detection may be effected in any convenient way, and for cell-free assays, one of the components usually comprises or is coupled to a label. Essentially any label can be used that provides for detection. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to a reagent or incorporated into the peptide structure, e.g. in the case of a peptide reagent, a methionine residue comprising a radioactive isotope of sulfur.

A variety of methods may be used to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

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For example, in the case of radioactive labels, emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters.

The assays of the invention are particularly suited to automated high throughput drug screening. In a particular embodiment, an automated mechanism, e.g. a mechanized arm, retrieves and transfers a microtiter plate to a liquid dispensing station where measured aliquots of each of an incubation buffer and a solution comprising one or more candidate agents are deposited into each designated well. The arm then retrieves and transfers to and deposits in designated wells a measured aliquot of a solution comprising a N-SMase protein or fragment or derivative thereof as well as solutions of other reagents such as a cleavage target. Thereafter, the arm transfers the microtiter plate to an analysis station where the reaction mixture can be analyzed for the presence or absence of various reaction products.

A preferred assay is disclosed in Example 2 which follows and which includes application of labeled sphingomyelin ( $[^{14}\text{C}]$ sphingomyelin) to a solid substrate such as a multi-well tray together with N-SMase.

As discussed above, N-SMase or fragments or derivatives thereof, and nucleic acids encoding same, also may be used for *in vitro* fertilization applications, particularly to improve viability and/or effective lifetime of sperm or seminal fluid samples during storage.

In this aspect, the invention also provides stored samples of human or other mammal such as cattle or horse sperm or seminal fluid in combination with an N-SMase fragment or derivative to thereby provide enhanced viability or lifetime of samples during the storage period.

For example, a sperm or seminal fluid storage unit of the invention may suitably comprise an N-SMase fragment or derivative of the invention in combination with a sperm or seminal fluid sample. That mixture also may optionally comprise a buffer or diluent as may be used with such samples. The N-SMase fragment or derivative preferably will be present in an amount sufficient to enhance the viability of lifetime of the sperm or seminal fluid sample during the storage period. Such storage effective amount amounts can be readily determined empirically for the particular N-SMase fragment or derivative employed. Storage effective amounts suitably may be at least

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about 0.01 weight percent of N-SMase derivative or fragment thereof based on total weight of the storage sample, more preferably at least about 0.05 weight percent. The sperm or seminal fluid sample may be human or of other mammal, e.g. horse, cattle or other livestock sample. The storage unit may suitably be a sterile cryovial or other  
5 vessel as conventionally employed to store sperm and seminal fluid.

The proteins and nucleic acids and fragments and derivatives thereof of the invention also may be used to generate immune responses. For example, it has been found that in certain instances where inappropriate expression of a self-protein is occurring an immune reaction can be useful. The nucleic acid permits the creation of  
10 unique peptides that can generate such a reaction. The characteristics needed to generate a peptide that will induce a MHC class I or II reaction are known and suitable peptides having such characteristics can be readily prepared based upon the present disclosure.

Antibodies also can be prepared that will bind to one or more particular  
15 domains of a peptide of the invention and can be used to modulate N-SMase activity.

Moreover, administration of an antibody against N-SMase or fragment or derivative thereof, preferably monoclonal or monospecific, to mammalian cells (including human cells) can reduce or abrogate TNF- $\alpha$  induced cell death (apoptosis) and the invention includes such therapeutic methods. In such methods, antibody  
20 against N-SMase can be administered to a mammal (including a human) by known procedures. It has been specifically found that apoptosis of human leukemic (HL-60) cells expressing N-SMase was abrogated by treatment with antibody against N-SMase.

The preferred therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of  
25 N-SMase or fragment or derivative thereof, or nucleic acid encoding same, to an animal in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from or susceptible to one of the above discussed disorders, including inflammatory disorders such as arthritis, osteoarthritis and Crohn's disease, obesity, diabetes, malignancies,  
30 particularly cancers including susceptible solid tumors, HIV, liver disorders including cirrhosis, excessive cholesterol levels, renal failure, cholesteryl ester storage disorder,

cardiac disease associated with LV dysfunction, atherosclerosis, undesired vascular restenosis, neurodegeneration, and central nervous system disorders such as depression, schizophrenia and Alzheimer's disease.

For therapeutic applications, peptides and nucleic acids of the invention may be suitably administered to a subject such as a mammal, particularly a human, alone or as part of a pharmaceutical composition, comprising the peptide or nucleic acid together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The pharmaceutical compositions of the invention include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes, and may be prepared by any methods well known in the art of pharmacy. See, for example, *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA (17th ed. 1985).

Such preparative methods include the step of bringing into association with the molecule to be administered ingredients such as the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or

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granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets optionally may be coated or scored and may be formulated so as to provide  
5 slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; and pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia.

10 Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented  
15 in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

20 Application of the subject therapeutics often will be local, so as to be administered at the site of interest. Various techniques can be used for providing the subject compositions at the site of interest, such as injection, use of catheters, trocars, projectiles, pluronic gel, stents, sustained drug release polymers or other device which provides for internal access. Where an organ or tissue is accessible because of removal  
25 from the patient, such organ or tissue may be bathed in a medium containing the subject compositions, the subject compositions may be painted onto the organ, or may be applied in any convenient way. Systemic administration of a nucleic acid using lipofection, liposomes with tissue targeting (e.g. antibody) may also be employed.

It will be appreciated that actual preferred amounts of a given peptide or  
30 nucleic acid of the invention used in a given therapy will vary to the particular active peptide or nucleic acid being utilized, the particular compositions formulated, the mode

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of application, the particular site of administration, the patient's weight, general health, sex, etc., the particular indication being treated, etc. and other such factors that are recognized by those skilled in the art including the attendant physician or veterinarian. Optimal administration rates for a given protocol of administration can be readily  
5 determined by those skilled in the art using conventional dosage determination tests.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1 - Cloning and expression of human neutral Sphingomyelinase

- 10 1. Purification of human neutral sphingomyelinase and preparation of antibodies.

Neutral sphingomyelinase (N-SMase) was prepared from human urine and polyclonal antibodies against that enzyme were raised in rabbits as described previously by S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989). Monospecific  
15 polyclonal antibodies against N-SMase were prepared by appropriate immuno-affinity absorption techniques as described in *J. Biol. Chem.*, 264:12,534-12,561 (1989).

2. Screening of cDNA library.

The human kidney library was purchased from Clontech (Palo Alto, CA) and was screened using anti-N-SMase described above according to the manufacturers  
20 protocol. Briefly, the  $\lambda$ gt11 phage was plated at  $3 \times 10^4$  pfu/150 mm plate on a lawn of E.Coli strain y1090r. Incubation was carried out at 42°C for 3.5 hrs to allow lytic phage growth. Then, a filter saturated with 10 mM IPTG was placed on top of the plate and incubated overnight at 37°C. Next, the filter was blocked with a solution of 5% non-fat dry milk for 1 hr at room temperature. Next, the filter was incubated with  
25 antibody against N-SMase at 1:200 dilution at room temperature overnight, and signal was detected by enhanced chemiluminescence technique (ECL, Amersham). Sixty-three clones were obtained by screening  $1 \times 10^6$   $\lambda$ gt11 phage clones. The most intense clones of cells were subjected to secondary and tertiary screening. All positive clones were subject to PCR to identify their insert size. Finally, a clone containing the longest  
30 insert (3.7 kb) referred to as  $\lambda^{32}$ -1 was used for further analysis by subcloning, sequencing and expression.



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### 3. Preparation of fusion proteins.

The protocol as described by the manufacturer's manual (Biotech, #5, 1992, pg. 636) was followed. By this procedure, the host growing in the logarithmic phase was infected with phage and incubated for 2 hr at 30°C. Next, IPTG (10 mM) was  
5 added and incubation continued at 37°C. The cell cultures were removed at 0, 15 min., 30 min., 45 min., 1 hr, 2 hr, 4 hr and 24 hr. The cells were centrifuged, washed with PBS, and stored frozen for N-SMase activity measurement.

### 4. Subcloning and sequencing of N-SMase cDNA.

First, the  $\lambda$  <sup>32</sup>-1 DNA was purified with the Magical Lambda preps DNA  
10 purification system from Promega by following the manufacturer's manual. Then, it was digested with the restriction endonucleosidase EcoRI. The 3.7kb insert was gel purified and subcloned into the EcoRI site of the vector pBluescriptII-SK (Stratagene, La Jolla, CA). The plasmid, thus generated was termed pBC32-2, and was purified using QIAGEN's DNA purification system. The N-SMase cDNA insert was  
15 sequenced with Sequenase using T7 and T3 primers by automatic sequence machine Model 373A (Applied Biosystems).

### 5. Transient Expression of N-SMase in Cos-7 cells.

To put N-SMase cDNA into a transient expression vector, the pBC32-2 was double digested with restriction endonucleosidases NotI and SalI. The 3.6 kb insert  
20 containing N-SMase was gel purified and inserted into a transient expression vector PSV-SPOT-1 (BRL). Thus, a plasmid called pHH<sub>1</sub> was constructed.

To transfect Cos-7 cells with pHH<sub>1</sub> and mock vector, 3 x 10<sup>5</sup> Cos-1 cells/plate in a p100 plate in 8 ml of Dulbecco's modified Eagle's medium (D-MEM) were seeded with 10% FCS (Bethesda Research Laboratory; BRL). Cells were incubated in a 10%  
25 CO<sub>2</sub> 37°C incubator until they are 80% confluent. The cells were then transfected with 10 µg of purified pHH<sub>1</sub> (QIAGEN) using LipoFectamine<sup>TM</sup> (BRL) in medium. Medium was changed after 5 hours of incubation. Finally, the cells were harvested at various time points (16 hr, 24 hr, 36 hr, and 48 hr, post transfection) by centrifugation at 1500 xg for 10 min., washed with phosphate buffered saline (PBS) and stored  
30 frozen at -20°C.

### 6. Activity gel assay of N-SMase expression in transfected Cos-7 cells.

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Both the bacterial cells and Cos-7 cells transfected with N-SMase cDNA were homogenized in Tris-glycine buffer (pH 7.4) containing 0.1% cutscum. The samples were mixed vigorously and sonicated for 10 sec. Next, the samples were transferred to a 4°C incubator and shaken for about 2 hours. Every hour, the samples were sonicated again on ice and further shaken. Subsequently, the samples were centrifuged at 10,000 xg for 10 min. The supernatants were collected, the protein content was measured and subjected to polyacrylamide gel electrophoresis using sodium laurylsarcosine. Subsequent to electrophoresis, the gel was sliced into several pieces and the activity of N-SMase was measured using [<sup>14</sup>C]-sphingomyelin as a substrate (see T. Taki and S. Chatterjee, *Analyt. Biochem.*, 224:490-493 (1995)).

7. Measurement of sphingomyelinase activity in aortic smooth muscle cells transiently transfected with N-SMase cDNA.

The activity of sphingomyelinase was measured in aortic smooth muscle cell extracts transiently transfected with pSVSPOT (mock cDNA) and pHH1 (cDNA for N-SMase) as shown previously in P. Ghosh and S. Chatterjee, *J. Biol. Chem.*, 262:12,550-12,556 (1987)). Briefly, the cell extracts (100 µg protein) were incubated with cutscum, MgCl<sub>2</sub>, human serum albumin and Tris glycine buffer (pH 7.4) plus [<sup>14</sup>C]-sphingomyelin (15,000 cpm) for 1 hr at 37°C. The reaction was terminated with 1 ml of 10% TCA and centrifuged. The supernatant was extracted with diethylether and the aqueous layer was withdrawn to measure radioactivity.

8. Reverse transcriptase-polymerase chain reaction.

Human kidney cells were seeded ( $1 \times 10^5$  cell/plate in p100 plates) and grown to confluence in complete medium with 10% fetal calf serum. Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction. 1 µg of total RNA was used to synthesize first strand cDNA using 20 pmol random hexamer primer, 200 units moloney murine leukemia virus reverse transcriptase, 0.5 mM each of deoxynucleotide triphosphates (Clontech, Palo Alto, CA) in buffer containing 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, and 0.5 units of RNase inhibitor, pH 8.3 at 42°C for 1 hour. 1 µl of 1:100 dilution of cDNA products was used to run PCR in 50 µl reaction mixture containing 0.2 mM each of dNTP, 2.0 units of Taq polymerase, 0.4 uM of each primers, 10 mM Tris HCl, 50 mM KCl, and 1.5 mM MgCl<sub>2</sub> (pH 8.3). The PCR was

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run for 35 cycles (30s at 94°C, 30s at 55°C and 30s at 72 °C) using a Perkin-Elmer Thermocycler.

9. Northern blot analysis.

One set of sequence primers from pBC32-2, T3-2/4T3R5, as RT-PCR primer  
5 was selected to conduct RT-PCR as described above. The 18-mer primers had the following sequence: TTGCGGCACTATTAGGTG (SEQ ID NO:5) and CGCCAATGCCAAAACGTA (SEQ ID NO:6). A 465 bp specific product was obtained, and gel purified. 50 ng of this product was labeled with 25  $\mu$ Ci[ $\alpha$ -<sup>32</sup>P] dATP and 25  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] dCTP using Random hexamer primers (BRL). The specific activity  
10 of this probe was  $1.88 \times 10^9$  cpm/ $\mu$ g. Next, the multiple tissue northern blot (Clontech; Palo Alto, CA) were hybridized with this probe(2x10) at 50°C overnight with hybridization buffer. Next, the blots were washed twice in 2x SSC, 0.05% SDS at room temperature for 30-40 min, then in 0.1 x SSC and 0.05% SDS for 40 min. at 50°C. Finally, the blot was exposed to an x-ray film at -70°C using two intensifying  
15 screens overnight.

10. Expression and purification of Glutathione-S-Transferase (GST)-NSMase fusion protein in E.Coli.

To prepare GST-NSMase fusion protein, an expression plasmid pJK2, pBC32-2 was digested with BssHII and EcoRI. A 2793 bp insert representing N-SMase open  
20 reading frame that is missing 18 bp N-terminal sequence, was ligated with a phosphorylated BamHI-BssHII linker containing the N-terminal sequence of GATCCATGATGACATATCACGAAACGCGCGTTTCGTGATA TGTCATCATG (SEQ ID NO:7) and a pGEX4T-1 vector double digested with BamHI and EcoRI (Pharmacia; Piscataway, NJ). To express and purify GST-N-SMase  
25 fusion protein, plasmid pJK2 was transformed into E.Coli (HB101) cells. A single colony of HB101 [pJK2] was grown in 2X YTA medium at 30°C until appropriate cell density ( $A_{600}=1.5$ ) was achieved. IPTG (0.1M) was added to induce fusion protein expression for 2 hours. Cells were harvested and the fusion protein was purified using Glutathione Sepharose-4B chromatography according to instructions provided by the  
30 manufacturer. N-SMase was released from the fusion protein by thrombin digestion.

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Such preparations were subjected to activity measurements and western immunoblot assays.

11. Coomassie blue staining and Western immunoblot assays of r-N-SMase.

20 µg of purified r-N-SMase was subjected to electrophoresis on 12.5% SDS-PAGE gel. One gel was stained with Coomassie blue according to standard protocol. Another gel were transferred onto a PVDF membrane. Next, the polyvinylidene difluoride (PVDF) membrane was blocked with 1% bovine serum albumin in TBS-T, incubated with anti-N-SMase at a dilution of 1:200 and developed with horse radish peroxidase (see S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989)).

12. Measurement of apoptosis in aortic smooth muscle cells transfected with cDNA for N-SMase.

Aortic smooth muscle cells transiently transfected with PVSPOT (mock cDNA) and pHH1 (cDNA for N-SMase) for 24 hours were solubilized and subjected to agarose gel electrophoresis (2 hr at 90 volts) for DNA fragmentation analysis.

Another set of cells transfected with cDNA were subjected to staining with the DNA-binding fluorochrome bis-benzimidine (Hoescht 323288; Sigma Chemical Co., St. Louis, MO). Briefly, transfected cells grown on glass cover slips were washed with PBS. The cells were fixed with 3% paraformaldehyde in PBS and incubated for 10 min at room temperature. The cells were washed with PBS and stained with 16 µg/ml of bis-benzimidine in PBS. After 15 min of incubation at room temperature, the samples were photographed. An Olympus BH2 fluorescence microscope with a BH2-DMU U2 UV mirror cube filter was used. Cells with three or more chromatin fragments were considered apoptotic.

The above-mentioned 3.7 kb nucleotide sequence of cDNA revealed an open reading frame size of 1197 base pairs which predicts a 397 amino acid polypeptide. The deduced amino acid sequence is shown in Figure 2. The estimated protein molecular weight is approximately 44 kDa, the estimated pI is 4.93. There are several potential modification sites in this protein: 1 N-glycosylation site at amino acid position 353; 1 tyrosine phosphorylation site at 238; and 2 cyclic AMP and cyclic GMP dependent protein kinase phosphorylation sites at position 218 and 357. It also has four casein kinase II phosphorylation sites at 3, 33, 65 and 101. Five

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myristoylation sites at 28, 44, 205, 206 and 220 bases were also found. There are ten protein kinase-C phosphorylation sites at 38, 48, 164, 216, 217, 236, 251, 260, 323 and 355.

Hydropathy plot analysis of N-SMase (Figure 3 of the drawings) indicates that there is no apparent transmembrane domain. The N-glycosylation site at amino acid position 353, tyrosine phosphorylation site at 238, as well as several other phosphorylation sites are presumably located on the exterior. Such sites may be subjected to further glycosylation and phosphorylation.

To confirm that the 3.7 kb cDNA does encode N-SMase with an apparent molecular weight of 44 kDa, constructs were made to fuse cDNA coding region with Glutathione-S-Transferase. Then, this plasmid was transformed into *E.coli* (HB101) to express and purify GST-NSMase fusion protein. The expression of fusion protein was induced by IPTG (0.1M) for 2 hours. The fusion protein was purified using Glutathione Sepharose-4B chromatography. The fusion protein has an apparent molecular weight of 73 kDa. After thrombin digestion, it resolved into two bands, 29 kDa and 44 kDa which correspond to GST and N-SMase, respectively. After N-SMase was released from fusion protein by Glutathione Sepharose-4B chromatography, it resolved as a single band, having a molecular weight on the order of 44 kDa. This protein was recognized by antibody against human N-SMase. See Figures 4A and 4B. Affinity-purified recombinant N-SMase expressed in *E.coli* had activity on the order of 3.9 nmole/mg protein/2h compared to mock cDNA transfected cells which had activity of 2.9 nmoles/mg protein/2h).

The results set forth in Figure 5 of the drawings show that Cos-7 cells transfected with pHHI exhibited a 10-fold increase in N-SMase activity compared to cells transfected with mock vector pSPOT-1 24 hours post-transfection. The most active recombinant N-SMase had an apparent molecular weight of 100 kDa. Significant N-SMase activity in a descending order was observed in protein bands having apparent kDa of 130 and 74. Those results indicate that N-SMase undergoes multiple post-translational modification.

The 465 bp RT-PCR fragment amplified 5' end of N-SMase cDNA coding region was used to probe N-SMase mRNA in various human tissues. N-SMase was

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expressed in all the human tissues investigated, the transcript size and copy numbers varied from one tissue to another (see Figure 6A of the drawings). The major transcript size of N-SMase expressed in all of these tissues is 1.7 kb. The other transcripts are 900 bp, 400 bp and 200 bp. Extended exposures of the x-ray film

5 showed additional transcripts on the order of 2.5 kb and 4.0 kb (Figure 6B) in human kidney. The cDNA of  $\lambda$  <sup>32</sup>-1 consists of 3670 bp, and contains a polyadenylation signal (ATTATT) at 351, (ATTAAA) at 805, (AATTAA) at 2562 and (ATTAAA) at 2835. These polyadenylation signals vary from the consensus AATAAA sequence, but it was found in 12% (ATTAAA) and 2% (AATTAA, ATTATT) of the mRNAs in

10 vertebrates, respectively. Such smaller transcripts exist due to their termination at different locations. The 1.7 kb transcript may be derived either from different genes or from alternative splicing.

Aortic smooth muscle cells transiently transfected with pHH1 (cDNA for N-SMase) had a 5-fold increase in enzyme activity compared to control (pSVSPOT) (see

15 Figure 7). This was accompanied by DNA fragmentation in pHH1 transfected cells and chromatin condensation. Based on an analysis of about 500 cells, about 32% of the cells were found to be apoptotic (see Figure 8C) as compared to control (Figure 8A) and mock cDNA transfected cells (Figure 8B). Those findings indicate that over-expression of N-SMase in aortic smooth muscle cells is accompanied by an increase in

20 enzyme activity and apoptosis.

#### EXAMPLE 2 - Protocol of a preferred N-SMase cleavage assay.

A. Reagents: N-methyl-<sup>14</sup>C]sphingomyelin (22,000 dpm/2  $\mu$ l in toluene:ethanol #:2 v/v). Cutsum (detergent) 0.002%, MgCl<sub>2</sub>, 20  $\mu$ g human serum albumin, 25  $\mu$ Mol Tris-glycine buffer pH 7.4. Enzyme (neutral sphingomyelinase) (1

25 ng - 1  $\mu$ g/well).

#### B. Preparation of Assay Plates:

First 2  $\mu$ l of [<sup>14</sup>C]sphingomyelin (22,000 dpm) is applied at the center of the PVDF well (Millipore MAIP-S-45-10), high protein binding Immobilon-P (0.4  $\mu$  thick in a 96 well plastic tray. The assay plates are dried in vacuum and stored until use.

30 C. Assay:

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The above reagents of Part A. are added to the well. Next, increasing concentration of the pure N-SMase (1 ng - 1 µg) or samples of enzyme (human fluids, cell extracts, etc.) are added. Then, the sphingomyelinase assay (incubation at 37°C for 30 minutes) is conducted. The contents of the reaction mixture are removed by  
5 suction attached to the bottom of the 96 well tray. After washing with PBS (5 times/ 50 µl) to remove non-specific radioactivity, 10 µl of liquid scintillation cocktail is added to each well and the [<sup>14</sup>C]sphingomyelin radioactivity which remains on the PVDF 96 well tray is counted in a Packard top β counter.

This assay can be completely automated employing modern robotic systems.  
10 This assay also enables high-throughput, e.g. 96 samples can be conveniently assayed for sphingomyelinase in 30 minutes. The assay also enables screening or identifying both inhibitors (antagonists) and activators (agonists) of N-SMase. The assay also may be employed to assay any enzyme that requires a lipid as a substrate that can be adsorbed to PVDF. The method also may be employed for antigen antibody binding  
15 assays, receptors binding assays, bacterial assays, viral assays and other toxin binding assays.

D. Controls for Assays:

Purified N-SMase (1 ng - 1 µg) is used as a standard control. Additionally, a constant set of samples (human urine) spiked with pure N-SMase are preferably  
20 employed and activity of such samples measured to serve as quality control and to assess day-to-day variation in the results.

All documents mentioned herein are incorporated herein by reference.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art,  
25 upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

- 28 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: Chatterjee, Subroto
- (ii) TITLE OF THE INVENTION: RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Dike, Bronstein, Roberts & Cushman, LLP
  - (B) STREET: 130 Water Street
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02109
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ Version 1.5
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/774,104
  - (B) FILING DATE: 12/24/96
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Corless, Peter F
  - (B) REGISTRATION NUMBER: 33,860
  - (C) REFERENCE/DOCKET NUMBER: 46906
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617-523-3400
  - (B) TELEFAX: 617-523-6440
  - (C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1197 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:



- 29 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

ATGATGACAT ATCACGAAAC GCGCGCGTTG GCTCAAAGCG ACTTACAGCA ACTCTATGCG 60
GCACTTGAAA CAACTGAATT TGGCGCTTAC TTTGCGACAC CCGCTGATGA TACTTTACGT 120
TTTGGCATTG GCGCAATCGC TACGGCAAAA ACGGCTCAGG CATTACAAGG TGCGGTTGTT 180
TTTGGTGCGC AGTCATTTGA TGAACAAGAG TACCCGCGAGT CTGAATTGAT GGCGGGTTTT 240
TGGTTTGTCC CCGAAGTGAT GGTGACCATC GCGGCAGATA AAATCACGTT CGGATCAGAT 300
ACCGTATCTG ATTTTACGAC GTGGCTGGCG CAGTTCGTGC CAAAACAGCC AAATACGGTG 360
ACCACTAGTC ATGTGACAGA TGAAGTGGAT TGGATCGAAC GGACAGAGAA TTTGATTGAT 420
ACCTTAGCCA TCGATCAAAC CTTAGCCAAA GTCGTTTTTG GTCGGCAACA GACCCTGCAG 480
TTATCCGACA CGTTACGACT GGCACAAATT ATTCGTGCGT TAGCTGAGCA GGCGAATACG 540
TATCATGTGG TTTTAAAGCG ACATGATGAA TTGTTTTATT CAGCAACACC GGAACGGTTA 600
GTGGCTATGT CAGGTGGTCA GATCGCTACG GCGGCGGTCT CTGGGACAAG CCGGCGCGGG 660
ACGGATGGCG CTGACGATAT CGCGTTAGGC GAAGCGTTGT TAGCCAGTCA GAAAACCCGC 720
ATTGAACATC AATATGTCGT GGCAAGTATC ACGACACGCT TGCAAGACGT GACGACGTCG 780
CTAAAGGTGC CGGCCATGCC AAGTTTACTC AAAAATAAGC AAGTTCAGCA TTTGTACACA 840
CCAATTACAG GGGACATTGC GGCACATTTA AGTGTGACCG CGATTGTTGA CCGCTTGCAT 900
CCAACACCAG CACTGGGTGG CGTCCCACGT GAAGCGGCCC TGTATTACAT TGCGACCCAT 960
GAGAAGACAC CTCGTGGCTT GTTTGCAGGT CCTATTGGCT ATTTTACCGC AGATAATAGT 1020
GGGGAATTTG TGGTTGGCAT CCGTTCCATG TATGTGAATC AAACGCAGCG ACGAGCAACT 1080
TTATTTGCTG GTGCCGGGAT TGTGGCTGAC TCCGATGCGC AACAAGAATA TGAAGAAACT 1140
GGGTTGAAAT TTGAACCCAT GCGGCAATTG TTAAAGGACT ACAATCATGT CGAATGA 1197

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 397 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Met Thr Tyr His Glu Thr Arg Ala Leu Ala Gln Ser Asp Leu Gln
1          5          10          15
Gln Leu Tyr Ala Ala Leu Glu Thr Thr Glu Phe Gly Ala Tyr Phe Ala
20          25          30
Thr Pro Ala Asp Asp Thr Leu Arg Phe Gly Ile Gly Ala Ile Ala Thr
35          40          45
Ala Lys Thr Ala Gln Ala Leu Gln Gly Ala Val Phe Gly Ala Gln Ser
50          55          60
Phe Asp Glu Gln Glu Tyr Pro Gln Ser Glu Leu Met Ala Gly Phe Trp
65          70          75          80
Phe Val Pro Glu Val Met Val Thr Ile Ala Ala Asp Lys Ile Thr Phe
85          90          95
Gly Ser Asp Thr Val Ser Asp Phe Thr Thr Trp Leu Ala Gln Phe Val
100          105          110
Pro Lys Gln Pro Asn Thr Val Thr Thr Ser His Val Thr Asp Glu Val
115          120          125
Asp Trp Ile Glu Arg Thr Glu Asn Leu Ile Asp Thr Leu Ala Ile Asp
130          135          140
Gln Thr Leu Ala Lys Val Val Phe Gly Arg Gln Gln Thr Leu Gln Leu

```

- 30 -

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     | 160 |
| Ser | Asp | Thr | Leu | Arg | Leu | Ala | Gln | Ile | Ile | Arg | Ala | Leu | Ala | Glu |
|     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |
| Ala | Asn | Thr | Tyr | His | Val | Val | Leu | Lys | Arg | His | Asp | Glu | Leu | Phe |
|     |     |     |     | 180 |     |     |     | 185 |     |     |     |     | 190 |     |
| Ser | Ala | Thr | Pro | Glu | Arg | Leu | Val | Ala | Met | Ser | Gly | Gly | Gln | Ile |
|     |     |     |     | 195 |     |     |     | 200 |     |     |     | 205 |     |     |
| Thr | Ala | Ala | Val | Ala | Gly | Thr | Ser | Arg | Arg | Gly | Thr | Asp | Gly | Ala |
|     |     |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |
| Asp | Ile | Ala | Leu | Gly | Glu | Ala | Leu | Leu | Ala | Ser | Gln | Lys | Asn | Arg |
| 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     | 240 |
| Glu | His | Gln | Tyr | Val | Val | Ala | Ser | Ile | Thr | Thr | Arg | Leu | Gln | Asp |
|     |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |
| Thr | Thr | Ser | Leu | Lys | Val | Pro | Ala | Met | Pro | Ser | Leu | Leu | Lys | Asn |
|     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |
| Gln | Val | Gln | His | Leu | Tyr | Thr | Pro | Ile | Thr | Gly | Asp | Ile | Ala | Ala |
|     |     |     | 275 |     |     |     | 280 |     |     |     |     | 285 |     |     |
| Leu | Ser | Val | Thr | Ala | Ile | Val | Asp | Arg | Leu | His | Pro | Thr | Pro | Ala |
| 290 |     |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |
| Gly | Gly | Val | Pro | Arg | Glu | Ala | Ala | Leu | Tyr | Tyr | Ile | Ala | Thr | His |
| 305 |     |     |     |     | 310 |     |     |     |     | 315 |     |     |     | 320 |
| Lys | Thr | Pro | Arg | Gly | Leu | Phe | Ala | Gly | Pro | Ile | Gly | Tyr | Phe | Thr |
|     |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |
| Asp | Asn | Ser | Gly | Glu | Phe | Val | Val | Gly | Ile | Arg | Ser | Met | Tyr | Val |
|     |     |     | 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |
| Gln | Thr | Gln | Arg | Arg | Ala | Thr | Leu | Phe | Ala | Gly | Ala | Gly | Ile | Val |
|     |     |     | 355 |     |     |     | 360 |     |     |     |     | 365 |     |     |
| Asp | Ser | Asp | Ala | Gln | Gln | Glu | Tyr | Glu | Glu | Thr | Gly | Leu | Lys | Phe |
| 370 |     |     |     |     | 375 |     |     |     |     |     | 380 |     |     |     |
| Pro | Met | Arg | Gln | Leu | Leu | Lys | Asp | Tyr | Asn | His | Val | Glu |     |     |
| 385 |     |     |     |     | 390 |     |     |     |     | 395 |     |     |     |     |

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ser Leu Lys Val Pro Ala  
 1 5

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ser Ile Thr Val Arg Val  
1 5

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGCGGCACT ATTAGGTG

18

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCAATGCC AAAACGTA

18

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 50 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCATGAT GACATATCAC GAAACGCGCG TTTCGTGATA TGTCATCATG

50

What is claimed is:

1. An isolated nucleic acid encoding human neutral sphingomyelinase.
2. The nucleic acid of claim 1 where the nucleic acid comprises the sequence of SEQ ID NO:1, or the complement thereto.
3. The nucleic acid of claim 1 where the nucleic acid codes for the human neutral sphingomyelinase of SEQ ID NO:2.
4. The nucleic acid of claim 1 where the human neutral sphingomyelinase has a molecular weight of about 44 kDa as determined by polyacrylamide gel electrophoresis using sodium laurylsarocine.
5. The nucleic acid of claim 1 where the nucleic acid has at least about 80 percent sequence identity to SEQ ID NO:1, or the complement thereto.
6. The nucleic acid of claim 1 wherein the polynucleotide is cDNA.
7. The nucleic acid of claim 1 wherein the polynucleotide is RNA.
8. A recombinant vector comprising the nucleic acid of claim 1.
9. A host cell comprising the vector of claim 8.
10. A method of producing human neutral sphingomyelinase comprising culturing a host cell of claim 9 under conditions suitable for expression of human neutral sphingomyelinase.
11. A nucleic acid that hybridizes to the sequence of SEQ ID NO:1 under normal stringency conditions.
12. The nucleic acid of claim 11 where the nucleic acid hybridizes to the sequence of SEQ ID NO:1 under high stringency conditions.
13. A method of identifying a compound useful in the diagnosis or treatment of a human neutral sphingomyelinase related disorder, comprising contacting a candidate pharmacological agent with human neutral sphingomyelinase or fragment or derivative thereof and analyzing the mixture of the candidate agent and human neutral sphingomyelinase or fragment or derivative thereof.
14. The method of claim 13 wherein the human neutral sphingomyelinase has a sequence represented by SEQ ID NO:2.

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15. The method of claim 13 wherein
- 1) a mixture is formed of
    - i) a human neutral sphingomyelinase cleavage target,
    - ii) the human neutral sphingomyelinase or fragment or derivative thereof, and
    - iii) a candidate pharmacological agent;
  - 2) the mixture is treated under conditions whereby, but for the presence of the candidate agent, the human neutral sphingomyelinase or fragment or derivative cleaves the cleavage target to yield a cleavage product; and
  - 3) the presence of the cleavage product is detected, wherein a reduced concentration of the cleavage product relative to a control mixture that does not contain the candidate agent identifies the candidate agent as a compound potentially useful in the diagnosis or treatment of a human neutral sphingomyelinase related disorder.
16. The method of claim 15 wherein the human neutral sphingomyelinase cleavage target is sphingomyelin.
17. The method of claim 15 wherein the human neutral sphingomyelinase cleavage product is ceramide.
18. An isolated human neutral sphingomyelinase having an apparent molecular weight of about 44 kDa as determined by polyacrylamide gel electrophoresis using sodium laurylsarcosine.
19. An isolated human neutral sphingomyelinase of claim 18 comprising a sequence represented by SEQ ID NO:2.
20. An isolated polypeptide having at least about 70 percent sequence identity to SEQ ID NO:2.
21. A method for modulating N-SMase activity comprising administering to human cells a modulation effective amount of a nucleic acid of claim 1 or fragment or derivative thereof.
22. A method for modulating N-SMase activity comprising administering to human cells a modulation effective amount of an isolated human neutral sphingomyelinase of claim 18 or fragment or derivative thereof.

23. A method for treating a disorder associated with N-SMase comprising administering to a patient suffering from or susceptible to such disorder an effective amount of an isolated nucleic acid of claim 1 or fragment or derivative thereof.

24. The method of claim 23 wherein the disorder is an inflammatory disorder, arthritis, osteoarthritis, Crohn's disease, obesity, diabetes, cirrhosis, susceptible tumors, central nervous system disorder, vascular restonsis, arterial occlusion arising from plaque formation, cardiac disease where LV dysfunction occurs, hypercholesteroloemia, cholesteryl ester storage disorder, renal failure, HIV infection, depression, schizophrenia, neurodegeneration and Alzheimer's disease.

25. A method for treating a disorder associated with N-SMase comprising administering to a patient suffering from or susceptible to such disorder an effective amount of an isolated human neutral sphingomyelinase of claim 18 or fragment or derivative thereof.

26. The method of claim 25 wherein the disorder is an inflammatory disorder, arthritis, osteoarthritis, Crohn's disease, obesity, diabetes, cirrhosis, susceptible tumors, central nervous system disorder, vascular restonsis, arterial occlusion arising from plaque formation, cardiac disease where LV dysfunction occurs, hypercholesteroloemia, cholesteryl ester storage disorder, renal failure, HIV infection, depression, schizophrenia, neurodegeneration and Alzheimer's disease.

27. A method of maintaining samples of sperm or seminal fluid comprising providing a mixture comprising sperm or seminal fluid and an effective amount of an a fragment or derivative of human neutral sphingomyelinase.

28. The method of claim 27 wherein the mixture comprises human sperm or seminal fluid.

29. A storage sample of sperm or seminal fluid comprising sperm or seminal fluid and a storage effective amount of a fragment or derivative of human neutral sphingomyelinase of claim 18.

30. A method to reduce TNF- $\alpha$  induced apoptosis of mammalian cells comprising administering to a mammal an effective amount of antibody against N-SMase or fragment or derivative thereof.

ATGATGACATATCACGAAACGGCGCGCTTGGCTCAAAGCGACTTACAGCA  
ACTCTATGCGGCACCTTGAACAACCTGAATTTGGCGCTTA  
CTTTGCGACACCCGCTGATGATACTTTACGTTTTGGCATTGGCGCAATCG  
CTACGGCAAAAACGGCTCAGGCATTACAAGTGCGGTTGTTTTGGTGCG  
CAGTCATTTGATGAACAAGAGTACCCGCAGTCTGAATTGATGGCGGGTTT  
TTGGTTTGTCCCCGAAGTGATGGTGACCATCGCGGCAGATAAAATCACGT  
TCGGATCAGATACCGTATCTGATTTTACGACGTGGCTGGCGCAGTTCGTG  
CCAAAACAGCCAAATACGGTGACCACTAGTCATGTGACAGATGAAGTGGA  
TTGGATCGAACGGACAGAGAATTTGATTGATACCTTAGCCATCGATCAA  
CCTTAGCCAAAGTCGTTTTTGGTCGGCAACAGACCCTGCAGTTATCCGAC  
ACGTTACGACTGGCACAAATTATTCGTGCGTTAGCTGAGCAGGCGAATAC  
GTATCATGTGGTTTTAAAGCGACATGATGAATTGTTTATTTAGCAACAC  
CGGAACGGTTAGTGGCTATGTCAGGTGGTCAGATCGCTACGGCGGCGGTC  
GCTGGGACAAGCCGGCGCGGGACGGATGGCGCTGACGATATCGCGTTAGG  
CGAAGCGTTGTTAGCCAGTCAGAAAAACCGCATTGAACATCAATATGTG  
TGGCAAGTATCACGACACGCTTGCAAGACGTGACGACGTGCTAAAGGTG  
CCGGCCATGCCAAGTTTACTCAAAAATAAGCAAGTTCAGCATTGTACAC  
ACCAATTACAGGGGACATTGCGGCACATTTAAGTGTGACCGCGATTGTTG  
ACCGCTTGCAATCCAACACCAGCACTGGGTGGCGTCCACGTGAAGCGGCC  
CTGTATTACATTGCGACCCATGAGAAGACACCTCGTGGCTTGTTCAGG  
TCCTATTGGCTATTTTACCGCAGATAATAGTGGGAATTTGTGGTTGGCA  
TCCGTTCCATGTATGTGAATCAAACGCAGCGACGAGCAACTTTATTTGCT  
GGTGCCGGGATTGTGGCTGACTCCGATGCGCAACAAGAATATGAAGAAAC  
TGGGTTGAAATTTGAACCCATGCGGCAATTGTTAAAGGACTACAATCATG  
TCGAATGA

FIGURE 1



1 M M T Y H E T R A L A Q S D L Q Q L Y A A L E T T E F G A Y F A T P A  
35 D D T L R F G I G A I A T A K T A Q A L Q G A V F G A Q S F D E Q E Y  
71 P Q S E L M A G F W F V P E V M V T I A A D K I T F G S D T V S D F T  
106 T W L A Q F V P K Q P N T V T T S H V T D E V D W I E R T E N L I D T  
141 L A I D Q T L A K V V F G R Q Q T L Q L S D T L R L A Q I I R A L A E  
176 Q A N T Y H V V L K R H D E L F I S A T P E R L V A M S G G Q I A T A  
211 A V A G T S R R G T D G A D D I A L G E A L L A S Q K N R I E H Q Y V  
246 V A S I T T R L Q D V T T S L K V P A M P S L L K N K Q V Q H L Y T P  
281 I T G D I A A H L S V T A I V D R L H P T P A L G G V P R E A A L Y Y  
316 I A T H E K T P R G L F A G P I G Y F T A D N S G E F V V G I R S M Y  
351 V N Q T Q R R A T L F A G A G I V A D S D A Q Q E Y E E T G L K F E P  
385 M R Q L L K D Y N H V E

FIGURE 2

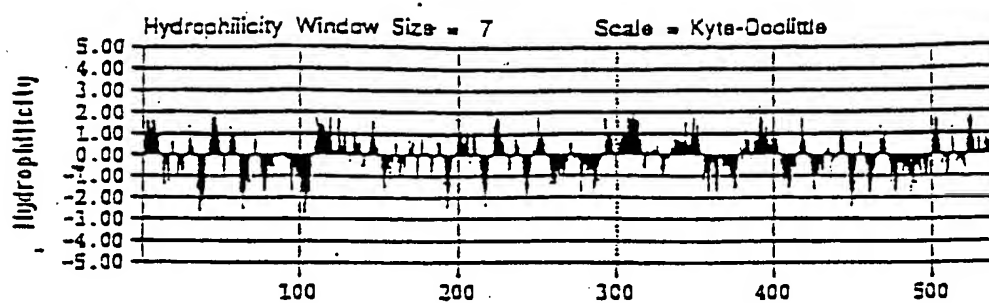


FIGURE 3

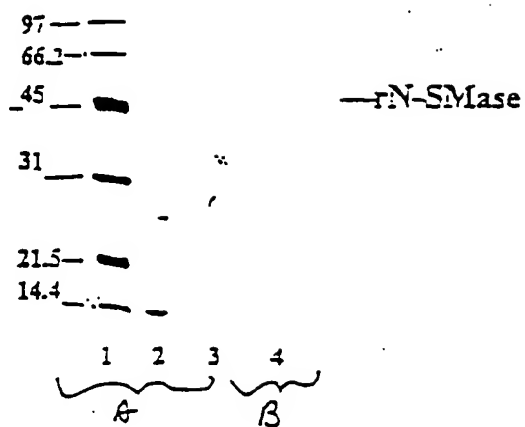


FIGURE 4A

FIGURE 4B

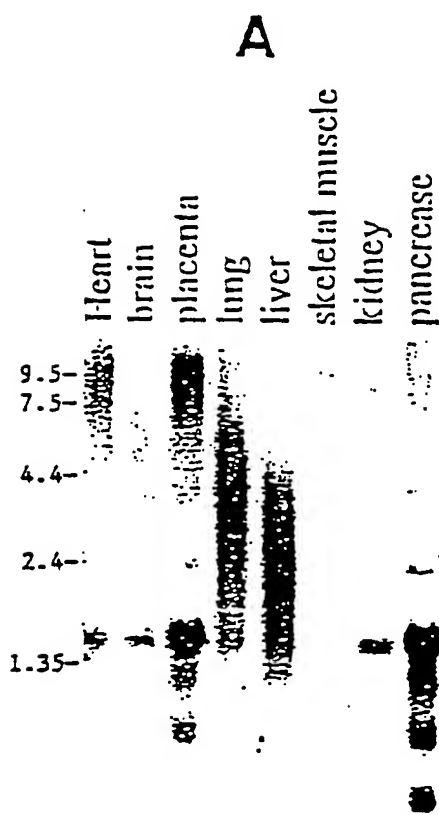


FIGURE 6A

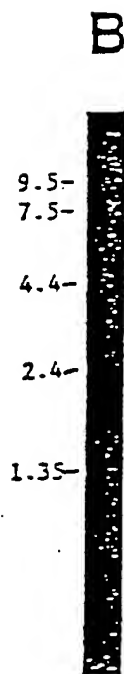


FIGURE 6B

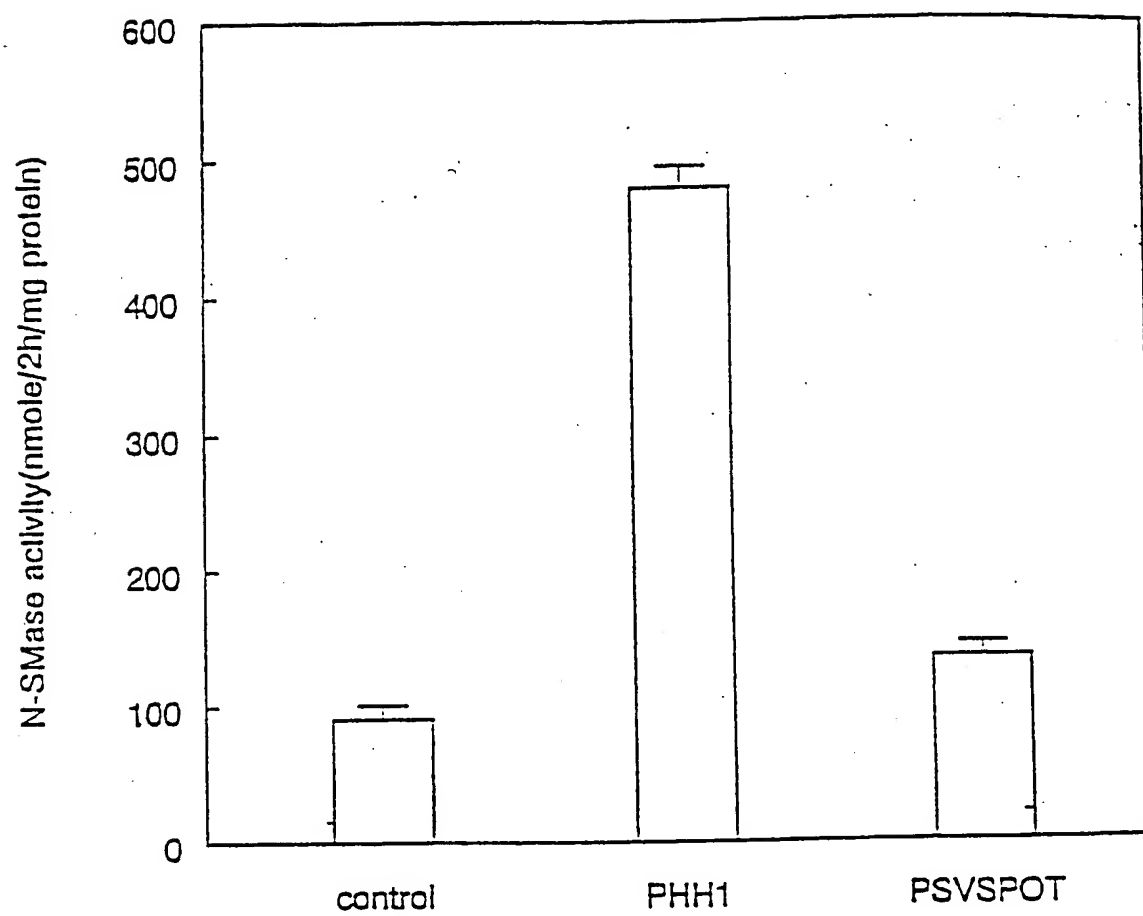


FIGURE 5

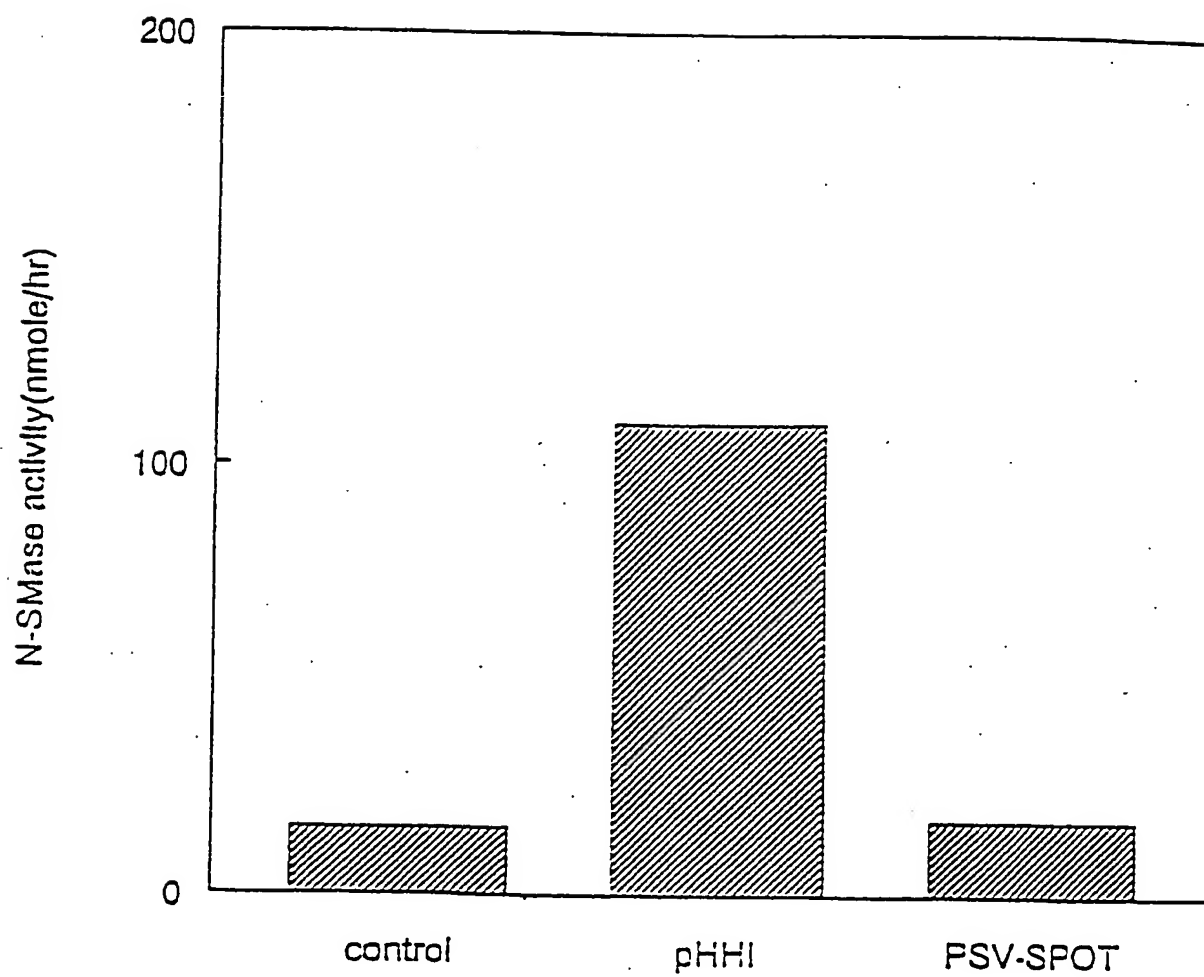


FIGURE 7

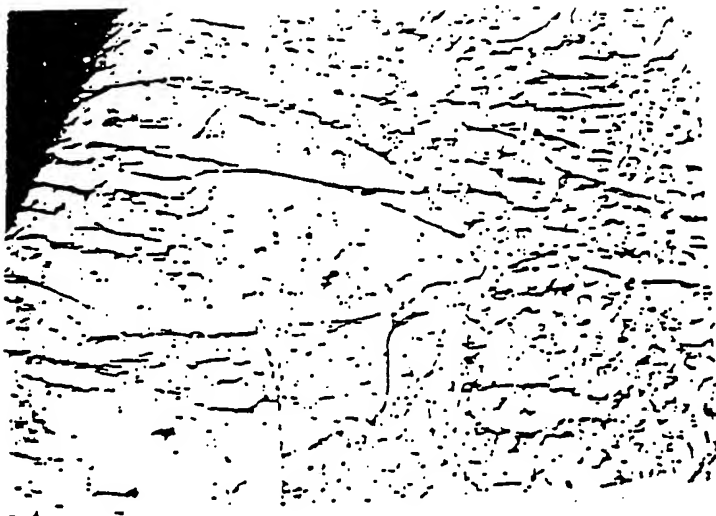


FIGURE 8A

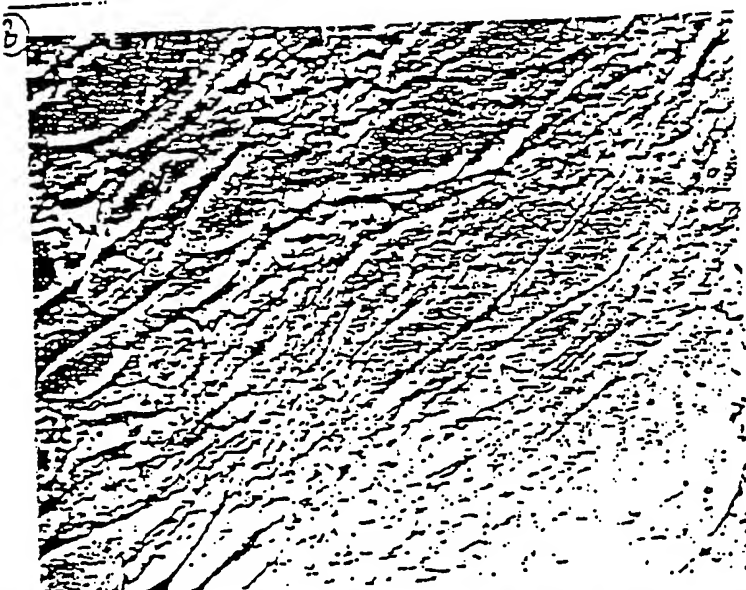


FIGURE 8B

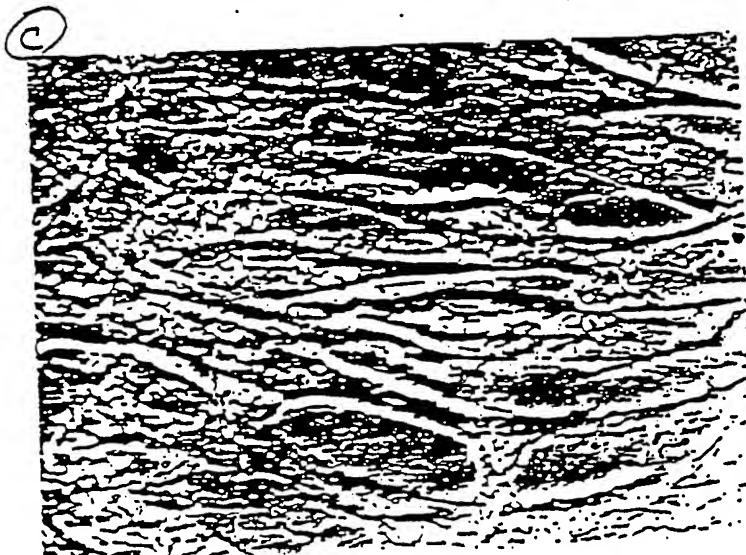


FIGURE 8C

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/24051

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12N 9/20, 9/22, 5/00, 1/20, 1/14, 15/00; C07H 21/04

US CL : 435/6, 198, 199, 252.3, 254.11, 325, 320.1; 536/23.2, 24.3

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 198, 199, 252.3, 254.11, 325, 320.1; 536/23.2, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and STN (Bioscience and Patent Indexes): neutral, sphingomyelinase#, sphingomyelin cholinephosphohydrolase#, human

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| Y         | CHATTERJEE ET AL. Neutral Sphingomyelinase from Human Urine. J. Biol. Chem. 25 July 1989. Vol. 264. No. 21. pages 12554-12561, see entire document.   | 1-12                  |
| Y         | IDA ET AL. Cloning of a Human Acid Sphingomyelinase cDNA with a New Mutation That Renders the Enzyme Inactive. J. Biochem. July 1993. Vol. 114. No. 1. pages 15-20, see entire document.                    | 1-12                  |
| Y         | QUINTERN ET AL. Isolation of cDNA Clones Encoding Human Acid Sphingomyelinase: Occurrence of Alternatively Processed Transcripts. EMBO J. August 1989. Vol. 8. No. 9. pages 2469-2473, see entire document. | 1-12                  |

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

|   |  |
|---|--|
| * Special categories of cited documents:  | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| *A* document defining the general state of the art which is not considered to be of particular relevance  | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| *B* earlier document published on or after the international filing date  | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| *P* document published prior to the international filing date but later than the priority date claimed  |  |

Date of the actual completion of the international search

27 FEBRUARY 1998

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 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

LISA J. ROBBES, PH.D.

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/24051

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-12

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/24051

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-12, drawn to nucleic acid molecules encoding human neutral sphingomyelinase, vectors, host cells and recombinant methods of production.

Group II, claims 13-17, drawn to methods of identifying compounds.

Group III, claims 18-20, drawn to human neutral sphingomyelinase.

Group IV, claims 21, 23 and 24, drawn to gene therapy methods.

Group V, claims 22, 25 and 26, drawn to methods of modulating enzymatic activity.

Group VI, claims 27-29, drawn to methods of maintaining samples.

Group VII, claim 30, drawn to methods of reducing apoptosis.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I has the special technical feature of the recombinant methods, which Groups II-VII do not share. Group II has the special technical feature of the methods for identifying molecules, which Groups I and III-VII do not share. Group III has the special technical feature of the enzyme, which Groups I-II and IV-VII do not share. Group IV has the special technical feature of gene therapy, which Groups I-III and V-VII do not share. Group V has the special technical feature of the methods of enzyme modulation, which Groups I-IV and VI-VII do not share. Group VI has the special technical feature of the methods of maintaining samples, which Groups I-V and VII do not share. Group VII has the special technical feature of the methods for reducing apoptosis, which Groups I-VI do not share.



(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES  
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(71) Anmelder (für alle Bestimmungsstaaten mit Aus-  
nahme von US): MEMOREC STOFFEL GMBH  
MEDIZINISCH-MOLEKULARE ENTWICKLUNG  
[DE/DE]; Stöckheimer Weg 1, D-50829 Köln (DE).

(22) Internationales Anmeldedatum:  
12. August 2000 (12.08.2000)

(72) Erfinder; und

(75) Erfinder/Anmelder (nur für US): HOFMANN, Kay  
[DE/DE]; Ehrenfeldgürtel 139, D-50823 Köln (DE).

(25) Einreichungssprache: Deutsch

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(74) Anwälte: MEYERS, Hans-Wilhelm usw.; Postfach 10 22  
41, D-50462 Köln (DE).

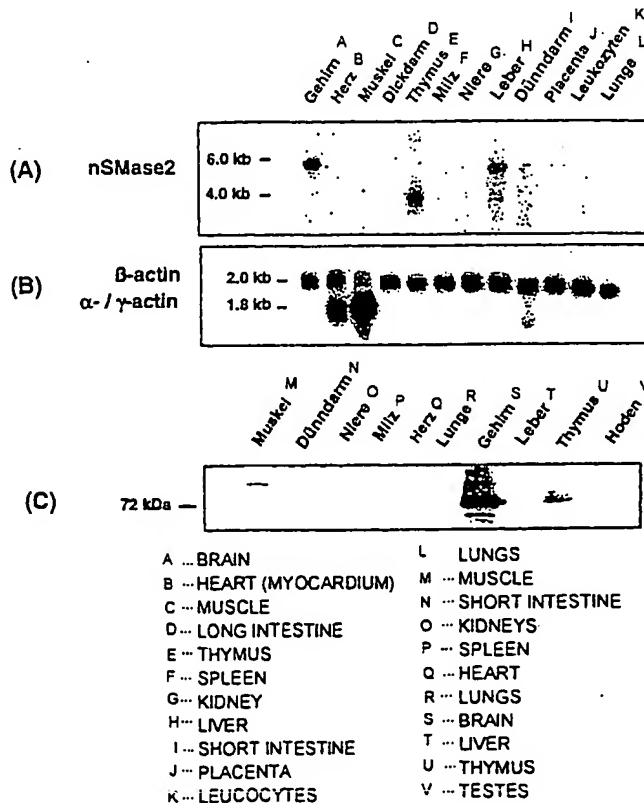
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(54) Title: NEUTRAL CEREBRAL-SPHINGOMYELINASE

(54) Bezeichnung: NEUTRALE HIRN-SPHINGOMYELINASE



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Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

**Veröffentlicht:**

- Mit internationalem Recherchenbericht.
- Vor Ablauf der für Änderungen der Ansprüche geltenden Frist; Veröffentlichung wird wiederholt, falls Änderungen eintreffen.

(57) Abstract: Nucleic acids which code for eucaryotic neutral cerebral-sphingomyelinase n (nSMase2) have the following sequence motifs:  $X_1-X_2-X_3-X_4-D-Y-X_5$  and  $X_6-X_7-T-D-H-X_8$ , whereby  $X_1, X_6 = A$  or  $G$ ;  $X_2, X_3 = R$  or  $K$ ;  $X_4, X_5, X_7, X_8 = I$  or  $L$  or  $V$  or  $M$ .

(57) Zusammenfassung: Nukleinsäure kodierend für eukaryontische neutrale Hirn-Sphingomyelinase (nSMase2) mit den Sequenzmotiven  $X_1-X_2-X_3-X_4-D-Y-X_5$  und  $X_6-X_7-T-D-H-X_8$ , wobei  $X_1, X_6 = A$  oder  $G$ ;  $X_2, X_3 = R$  oder  $K$ ;  $X_4, X_5, X_7, X_8 = I$  oder  $L$  oder  $V$  oder  $M$  ist.

### **Neutrale Hirn-Sphingomyelinase**

Die vorliegende Erfindung betrifft Nukleinsäuren, die für humane oder murine eukaryontische neutrale Hirn-Sphingomyelinase codieren, und ihre Anwendung.

Sphingomyelin ist eine wesentliche Komponente von Plasmamembranen. Der Abbau des Sphingomyelins gibt eine Vielzahl von Substanzen, die potentiell second messenger Eigenschaften haben, z.B. Ceramid, Sphingosin, Sphingosin-1-phosphat. Es sind zwei sphingomyelinspaltende Enzymaktivitäten bekannt, zum einen die der lysosomalen sauren Sphingomyelinase und zum anderen die der membran-gebundenen neutralen Sphingomyelinase.

Im Menschen und in Säugetieren liegt die Aktivität der neutralen Sphingomyelinase bevorzugt im Gehirn vor. Die einzige bisher charakterisierte eukaryontische neutrale Sphingomyelinase kommt in Säugern in allen Gewebetypen vor und ist nicht maßgeblich für die im Gehirn messbare Sphingomyelinase-Aktivität verantwortlich.

Durch die vorliegende Erfindung werden erstmals Nukleinsäuren, codierend für humane oder murine eukaryontische neutrale Hirn-Sphingomyelinase, verfügbar gemacht. Sie wird auch als nSMase2 bezeichnet. Die eukaryontische neutrale Hirn-Sphingomyelinase ist dadurch charakterisiert, dass sie im Gehirn angereichert ist, Sphingomyelin in Ceramid und Phosphocholin spaltet und die Aktivität von der Zugabe von Magnesiumionen abhängig ist. Es handelt sich um ein membran-gebundenes Enzym. Die maximale Aktivität wird im neutralen pH-Bereich erzielt.

Das Molekulargewicht liegt im Bereich von 50 bis 80 kD, vorzugsweise im Bereich von 70 bis 75 kD.

Figur 1 zeigt die Ergebnisse von Northern- und Westernblots aus verschiedenen humanen Geweben.

Bevorzugt handelt es sich bei der erfindungsgemäßen Nukleinsäure um Nukleinsäuren mit der Seq. ID. Nr. 3 und Seq. ID. Nr. 4.

Die erfindungsgemäßen Nukleinsäuren eignen sich zur Expression der eukaryontischen neutralen Hirn-Sphingomyelinase in pro- oder eukaryontischen Systemen. Darüber hinaus sind sie auch zur Expression der nSMase2 in vivo im Sinne einer Gentherapie oder insbesondere in Form von Fragmenten auch in komplementärer Struktur als Antisense-Nukleotide zur Verringerung der Expression der nSMase2 geeignet.

Die erfindungsgemäßen Nukleinsäuren können durch chemische Synthese oder durch Vervielfältigung in gentechnisch veränderten Organismen nach dem Fachmann an sich bekannten Verfahren hergestellt werden.

Gegenstand der Erfindung ist auch die durch die Expression der erfindungsgemäßen Nukleinsäuren erhältliche eukaryontische neutrale Hirn-Sphingomyelinase.

Sie weist zwei Teilmotive  $X_1-X_2-X_3-X_4-D-Y-X_5$  und  $X_6-X_7-T-D-H-X_8$ , wobei  $X_1, X_6 = A$  oder  $G$ ;  $X_2, X_3 = R$  oder  $K$ ;  $X_4, X_5, X_7, X_8 = I$  oder  $L$  oder  $V$  oder  $M$  ist, auf.

Die erfindungsgemäße nSMase2 lässt sich durch Expression in gentechnisch veränderten Organismen herstellen. Insbesondere sind eukary-

ontische Expressionssysteme geeignet. Entsprechende eukaryontische Expressionssysteme sind dem Fachmann bekannt wie beispielsweise pRc/CMV (Firma Stratagene). Die Aufreinigung aus gentechnisch veränderten Organismen bietet, insbesondere im Falle der Überexpression, einen leichten und direkten Zugang zur erfindungsgemäßen nSMase2 und erlaubt darüber hinaus die Isolierung in größeren Mengen.

Die Aminosäuresequenzen der humanen und murinen neutralen Hirn-Sphingomyelinase sind als Seq. ID. Nr. 1 und 2 wiedergegeben.

Die Molekulargewichte der humanen bzw. murinen Sphingomyelinase 2 beträgt jeweils etwa 71 kDa. Im Gegensatz zu den bakteriellen nSMasen enthalten die erfindungsgemäßen nSMase2 Sequenzen keine Signalsequenz am N-Terminus. Aufgrund der Hydrophobizitätsanalyse kann davon ausgegangen werden, dass zwei benachbarte hydrophobe Membrandomänen am N-Terminus durch 35 Aminosäuren getrennt sind. Es scheint sich daher um integrale Membranproteine zu handeln, deren katalytisch aktive Domäne zum Cytosol zeigt, während nur ein geringer Anteil der Enzyme Kontakt zur extrazellulären Umgebung hat. Dies ist im Gegensatz zu den bakteriellen nSMasen, bei denen es sich um sekretierte, lösliche Proteine handelt, ist aber in Übereinstimmung mit bisherigen Untersuchungen zu den Eigenschaften der neutralen Sphingomyelinasen von Säugetieren. Die ubiquitäre eukaryontische Sphingomyelinase nSMase1 besitzt ebenfalls zwei hydrophobe Transmembrandomänen, die sich jedoch am C-Terminus des Proteins befinden.

Die 6 kb mRNA der humanen nSMase2 wird gemäß Northern Blot Analyse bevorzugt im Gehirn exprimiert. In Leber und Dünndarm zeigt der Northern Blot ein schwächeres Signal, während in Thymus eine kreuzhybridisierende mRNA von unterschiedlicher Größe (3.5 kB) exprimiert wird.

Das pH-Optimum der erfindungsgemäßen neutralen Hirn-Sphingomyelinase liegt im Bereich von 6 bis 8. Die Aktivität ist magnesiumionenabhängig, die Zugabe von EDTA führt zu einer Inhibierung der nSMase-Aktivität, kann jedoch durch Zugabe von  $Mn^{2+}$ - oder  $Mg^{2+}$ -Ionen wiederhergestellt werden. Die Aktivität ist unbeeinflusst durch Behandlung mit DTT oder 2-Mercaptoethanol.

Weiterhin beansprucht werden Varianten der eukaryontischen neutralen Hirn-Sphingomyelinase. Unter den Begriff "Varianten" fallen sowohl natürlich vorkommende allelische Variationen der eukaryontischen neutralen Hirn-Sphingomyelinase sowie durch rekombinante DNA-Technologie (insbesondere durch in vitro Mutagenese mit Hilfe von chemisch synthetisierten Oligonukleotiden) und anschließende Expression erzeugte Proteine, die hinsichtlich ihrer biologischen und/oder immunologischen Aktivität der eukaryontischen neutralen Sphingomyelinase entsprechen. Dabei können sowohl Aminosäuren deletiert, eingefügt oder konservativ ausgetauscht werden. Konservativer Austausch bedeutet, daß eine Aminosäure durch eine Aminosäure ersetzt wird, die ähnliche physikalisch-chemische Eigenschaften aufweist.

So sind beispielsweise folgende Aminosäuren austauschbar: Serin für/gegen Alanin, Alanin für/gegen Glycin, Methionin für/gegen Serin, Lysin für/gegen Arginin, Lysin für/gegen Serin.

Insbesondere umfasst der Begriff Varianten auch N- und/oder C-terminale verkürzte Proteine sowie acetylierte, glykosylierte, amidierter und/oder phosphorylierte Derivate. Auch Verbindungen, bei denen nSMase2 oder ihre Varianten mit weiteren Molekülen wie Farbstoffen, Radionukliden oder Affinitätskomponenten gekoppelt sind, stellen erfindungsgemäße Varianten dar.



Beansprucht werden auch Nukleinsäuren, die für eukaryontische neutrale Hirn-Sphingomyelinase codieren bzw. komplementär zu diesen Nukleinsäuren sind. Bei den Nukleinsäuren kann es sich beispielsweise um DNA, RNA, PNA oder um nukleaseresistente Analoga handeln. Nukleaseresistente Analoga sind insbesondere solche Verbindungen, in denen die Phosphodiesterbindung durch hydrolysestabile Verbindungen modifiziert sind, beispielsweise Phosphothioate, Methylphosphonate o.ä.

Für Antisensenukleotide sind insbesondere kurze Fragmente der Nukleinsäuren geeignet. Diese sollten aus Gründen der Spezifität bevorzugt mehr als 6, noch mehr bevorzugt mehr als 8 und am meisten bevorzugt mehr als 12 Nukleotide aufweisen. Aus Gründen der Diffusion und der Kosten haben sie üblicherweise eine Länge von weniger als 30 Nukleotiden, bevorzugt 24 oder weniger und noch mehr bevorzugt 18 oder weniger Nukleotide.

Gegenstand der Erfindung sind auch Derivate von Nukleinsäuren, die für diagnostische oder therapeutische Zwecke mit anderen Molekülen gekoppelt sind, beispielsweise mit Fluoreszenzfarbstoffen, radioaktiven Markern oder Affinitätskomponenten, sowie Fragmente der erfindungsgemäßen Nukleinsäuren und der zu diesen Nukleinsäuren komplementären Nukleinsäuren sowie Varianten der Nukleinsäuren.

Fragmente bezeichnet dabei Nukleinsäuren, die am 5' oder 3' oder an beiden Seiten verkürzt sind. Unter dem Begriff "Varianten" wird verstanden, dass diese Nukleinsäuren unter stringenten Bedingungen mit der erfindungsgemäßen Nukleinsäure bzw. dazu komplementären Nukleinsäuren hybridisieren. Unter dem Begriff "stringente Bedingungen" wird verstanden, dass die Hybridisierung bei Bedingungen durchgeführt wird, bei der die Temperatur noch bis zu 10°C unter der Temperatur liegt (bei

sonst identischen Bedingungen), bei der exakt komplementäre Nukleinsäuren gerade noch hybridisieren würden. Wenn beispielsweise eine exakt hybridisierende Nukleinsäure unter gegebenen Bedingungen bis zu einer Temperatur von ca. 55°C hybridisiert, dann sind stringente Bedingungen Temperaturen gleich oder höher 45°C. Bevorzugt ist der Temperaturbereich für stringente Bedingungen von 5°C, noch mehr bevorzugt von 3°C.

Desweiteren betrifft die Erfindung Antikörper, die gegen die erfindungsgemäße nSMase2 oder die erfindungsgemäßen Nukleinsäuren gerichtet sind. Diese Substanzen eignen sich insbesondere zum Einsatz in der Diagnostik, dem Fachmann an sich bekannten Immunoassays, zur histologischen Untersuchung sowie als Arzneimittel zur Behandlung von Zuständen, die mit einer Überexpression der nSMase verbunden sind. Solche erfindungsgemäßen Antikörper können mit dem Fachmann an sich bekannten Verfahren durch Immunisierung mit nSMase, erfindungsgemäßen Nukleinsäuren oder Peptid- und Nukleinsäurenfragmenten in Gegenwart von Hilfsreagenzien erhalten werden.

Weiterhin sind Gegenstand der Erfindung Zelllinien, die die erfindungsgemäße nSMase2 überexprimieren. Solche Zelllinien sind erhältlich durch Transfektion mit Vektoren, die die erfindungsgemäßen Nukleinsäuren, die für nSMase kodieren, enthalten. Im Falle von eukaryontischen Zelllinien kann die Transfektion beispielsweise durch Elektroporation erfolgen. Die Zelllinien sind dabei vorzugsweise stabiltransfiziert.

Überexpression bedeutet in diesem Zusammenhang, dass diese Zelllinie eine höhere Aktivität der nSMase aufweisen als die Zelllinien, die nicht mit den erfindungsgemäßen Nukleinsäuren transfiziert wurden. Geeignete eukaryontische Zelllinien sind beispielsweise die Zelllinien U937, HEK 293 oder Jurkat.

Die Zelllinien zeigten in Experimenten eine spezifische nSMase-Aktivität zwischen 0,1 und 10  $\mu\text{mol/mg Protein/Stunde}$ .

Figur 1 zeigt die Northern- und Westernblot Analyse der nSMase2-Expression in humanen Geweben. Teil A zeigt dabei das Ergebnis einer Northernblot Hybridisierung mit einer nSMase2 Sonde. Teil B zeigt als Kontrolle das Ergebnis einer Northernblot Hybridisierung mit einer Sonde spezifisch für Actin. Teil C zeigt einen Westernblot mit einem Antikörper spezifisch für rekombinant hergestellte nSMase2.

Die Figuren 2 bis 5 zeigen die erfindungsgemäßen Sequenzen.

Die Zugabe von 0,5 mM Arachidonsäure führte zu einer dreifachen Erhöhung der nSMase-Aktivität in einem Extrakt aus HEK 293 Zellen, die nSMase2 überexprimieren. Die Zugabe von Phosphatidylserin führte zu einer sechsfachen Erhöhung der nSMase-Aktivität in einem Extrakt aus HEK 293 Zellen, die nSMase2 überexprimieren.

Gegenstand der Erfindung ist weiterhin ein transgenes Säugetier, das eine Überexpression (gain of function) oder eine Gendefizienz bzw. einen Gendefekt (loss of function) für die erfindungsgemäße nSMase2 aufweist. Bevorzugt handelt es sich bei dem Säugetier um ein Nagetier, insbesondere eine Maus. Diese transgenen Säugetiere sind durch für den Fachmann an sich bekannte Verfahren erhältlich und eignen sich insbesondere zur Funktionsaufklärung der neutralen Sphingomyelinase. Für transgene Säugetiere werden definierte Genkonstrukte durch DNA-Mikroinjektion in den Vorkern (Pronukleus) einer befruchteten Eizelle im Einzellstadium injiziert, um die Expression des zusätzlichen Gens zu erreichen. Durch zielgerichtete Veränderung eines Gens im Genoms von ES-Zellen, die nachfolgend in Blastozysten injiziert werden, wird die Funktion eines Gens

ausgeschaltet.

Bevorzugt handelt es sich bei den transgenen Tieren um Tiere, bei denen das Gen zeitlich und gewebspezifisch von außen induzierbar ein- bzw. ausgeschaltet werden kann. Entsprechende transgene Säugetiere eignen sich insbesondere zur Aufklärung der mit der erfindungsgemäßen nSMase im Zusammenhang stehenden Stoffwechsel- und Signaltransduktionswegen, die wiederum diagnostische oder therapeutische Anwendungen eröffnen. Insbesondere eignen sich die transgenen Säugetiere zum Screening von pharmazeutischen Wirkstoffen.

Die erfindungsgemäße eukaryontische neutrale Sphingomyelinase, die erfindungsgemäßen Nukleinsäuren sowie die erfindungsgemäßen Antikörper können in Arzneimitteln und Diagnostikmitteln gegebenenfalls zusammen mit weiteren Hilfsstoffen enthalten sein. Diese Arznei- und Diagnostikmittel eignen sich zur Diagnose und Behandlung von Erkrankungen, die auf einer Über- oder Unterexpression und/oder einer erhöhten oder verminderten Aktivität der eukaryontischen neutralen Sphingomyelinase und/oder auf Störungen der Zellproliferation, Zelldifferenzierung und/oder Apoptose beruhen.

Insbesondere sind dies Erkrankungen, bei denen Entzündungsprozesse, Zellwachstumstörungen und Stoffwechselstörungen eine Rolle spielen. Dies können beispielsweise Krebserkrankungen oder Störungen der Cholesterinhomöostase (Arteriosklerose) sein.

Ein erfindungsgemäßes pharmazeutisches Screening-Verfahren beruht auf der Veränderung der Expression oder Aktivität der erfindungsgemäßen nSMase2 in nSMase2-überexprimierenden Zelllinien bei Zugabe von mindestens einer potentiell pharmazeutisch wirksamen Substanz. Die

Zelllinien eignen sich somit insbesondere zur Entwicklung und Prüfung von pharmazeutischen Leitstrukturen.

Die Erfindung soll durch die folgenden Beispiele weiter erläutert werden.

### **Beispiel 1**

#### *Klonierung der Nukleinsäure*

Die erfindungsgemäßen für die neutrale Hirn-Sphingomyelinase kodierenden Nukleinsäuren wurden in die NotI Schnittstellen der Klonierungsstelle des eukaryontischen Expressionsvektors pRc/CMV (Stratagene) kloniert. Die erhaltenen Sequenzen wurden durch Sequenzierung mit einem Perkin-Elmer DNA-Sequencer 377A erhalten.

### **Beispiel 2**

#### *Klonierung der RNA*

Die Gesamt-RNA wurde nach bekannten Methoden aus verschiedenen Organen von acht drei Wochen alten CD1 Mäusen isoliert und Poly(A<sup>+</sup>)-RNA wurde durch Affinitätsreinigung an Oligo(dT)cellulose (Boehringer Mannheim Deutschland) gemäß Standardmethoden isoliert.

### **Beispiel 3**

#### *Überexprimierende Zelllinien*

HEK 293 Zellen wuchsen in DMEM Medium mit 10% fötalem Kälberserum; 1 µg/ml Penicillin/Streptomycin und 1mM Pyruvat bei 37°C und 5% CO<sub>2</sub>. 5x10<sup>6</sup>-Zellen wurden mit 1 µg linearisierter Plasmid-DNA, die für die erfindungsgemäße nSMase kodierte durch Elektroporation mit einem "gene pulser" (Firma Bio-Rad) transfiziert. Die Selektion stabiler Klone

erfolgte unter 1 mg/ml Geneticin (G418, Life Technologies, Gaithersburg, MD).

Die aus den Zelllinien aufgereinigte nSMase zeigte eine spezifische Aktivität zwischen 0,2 und 1  $\mu\text{mol/mg Protein/Stunde}$ . Das pH-Optimum lag bei 6 und 8. Die Aktivität war von der Anwesenheit von Magnesiumionen abhängig; die Zugabe von EDTA inhibierte die Aktivität.

#### **Beispiel 4**

##### *Messung der nSMase2-Aktivität*

Die enzymatische Aktivität wurde in Zellen und Mäusegewebe untersucht. Die Zellen wurden zweimal mit eiskaltem PBS gewaschen und bei 1.000 g sedimentiert. Das Pellet wurde in Lysepuffer resuspendiert und die Zellen wurden durch wiederholtes Einfrieren und Auftauen zerstört. Nach Zentrifugation für 2 min bei 2.500 g gefolgt von einer Extraktion mit Lysepuffer mit 0,2% Triton X-100. Anschließend erfolgt eine Zentrifugation für 15 min bei 100.000 g.

Gewebe von drei Wochen alten Mäusen wurde in kaltem Lysepuffer homogenisiert. Die zu untersuchende Menge an Protein oder homogenisiertem Gewebe wurde mit 10 nm (80.000 dpm)  $[\text{N-}^{14}\text{CH}_3]$ -Sphingomyelin für 30 min bei 37°C in einem Gesamtvolumen von 200  $\mu\text{l}$  inkubiert. Dann wurden 100  $\mu\text{l}$  Wasser zugesetzt und unreaktiertes Substrat durch Extraktion mit Chloroform-Methanol (2:1, v/v) entfernt. Die Radioaktivität der wässrigen Phase, die das enzymatisch freigesetzte Phosphocholin enthielt, wurde in einem Szintillationszähler gemessen.

### Patentansprüche

1. Nukleinsäure kodierend für eukaryontische neutrale Hirn-Sphingomyelinase (nSMase2) mit den Sequenzmotiven  $X_1$ - $X_2$ - $X_3$ - $X_4$ -D-Y- $X_5$  und  $X_6$ - $X_7$ -T-D-H- $X_8$ , wobei  $X_1$ ,  $X_6$  = A oder G;  $X_2$ ,  $X_3$  = R oder K;  $X_4$ ,  $X_5$ ,  $X_7$ ,  $X_8$  = I oder L oder V oder M ist.
2. Nukleinsäure nach Anspruch 1, dadurch gekennzeichnet, dass es sich um neutrale Hirnsphingomyelinase eines Säugetiers, insbesondere um murine oder humane Hirnsphingomyelinase handelt.
3. Nukleinsäure gemäß Anspruch 1 oder 2, dadurch gekennzeichnet, dass die neutrale Sphingomyelinase Sphingomyelin in Ceramid und Phosphocholin spaltet und ihre Aktivität von der Zugabe von Magnesiumionen abhängig ist.
4. Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 3 mit der Sequenz gemäß Seq. ID. Nr. 3 oder Seq. ID. Nr. 4.
5. Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 4 dadurch gekennzeichnet, dass es sich um DNA, RNA, PNA oder nukleaseresistente Analoga handelt.
6. Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 5 dadurch gekennzeichnet, daß es sich um mRNA, cDNA oder genomische DNA handelt.

7. Nukleinsäure dadurch gekennzeichnet, dass sie komplementär zur Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 6 ist.
8. Eukaryontische neutrale Hirnsphingomyelinase erhältlich durch Expression der Nukleinsäure gemäß Anspruch 1 bis 7, insbesondere mit der Sequenz gemäß Seq. ID. Nr. 1 oder Seq. ID. Nr. 2.
9. Antikörper, dadurch gekennzeichnet, dass sie gegen eukaryontische neutrale Hirnsphingomyelinase gemäß Anspruch 8 oder eine Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 7 gerichtet sind.
10. Zelllinie, dadurch gekennzeichnet, dass sie eukaryontische neutrale Hirnsphingomyelinase gemäß Anspruch 8 überexprimiert.
11. Zelllinie gemäß Anspruch 10, dadurch gekennzeichnet, dass es sich um eine eukaryontische neutrale Hirn-Sphingomyelinase exprimierende Zelllinie handelt, die auf den Zelllinien U937, HEK 293 oder Jurkat beruht.
12. Transgenes Säugetier mit Überexpression (gain of function) oder Gendefizienz oder Gendefekt (loss of function) für eukaryontische neutrale Hirn-Sphingomyelinase.
13. Transgenes Säugetier gemäß Anspruch 12, dadurch gekennzeichnet, dass es ein Nagetier ist.
14. Arzneimittel enthaltend eukaryontische neutrale Hirn-Sphingomyelinase gemäß Anspruch 8, eine Nukleinsäure gemäß min-

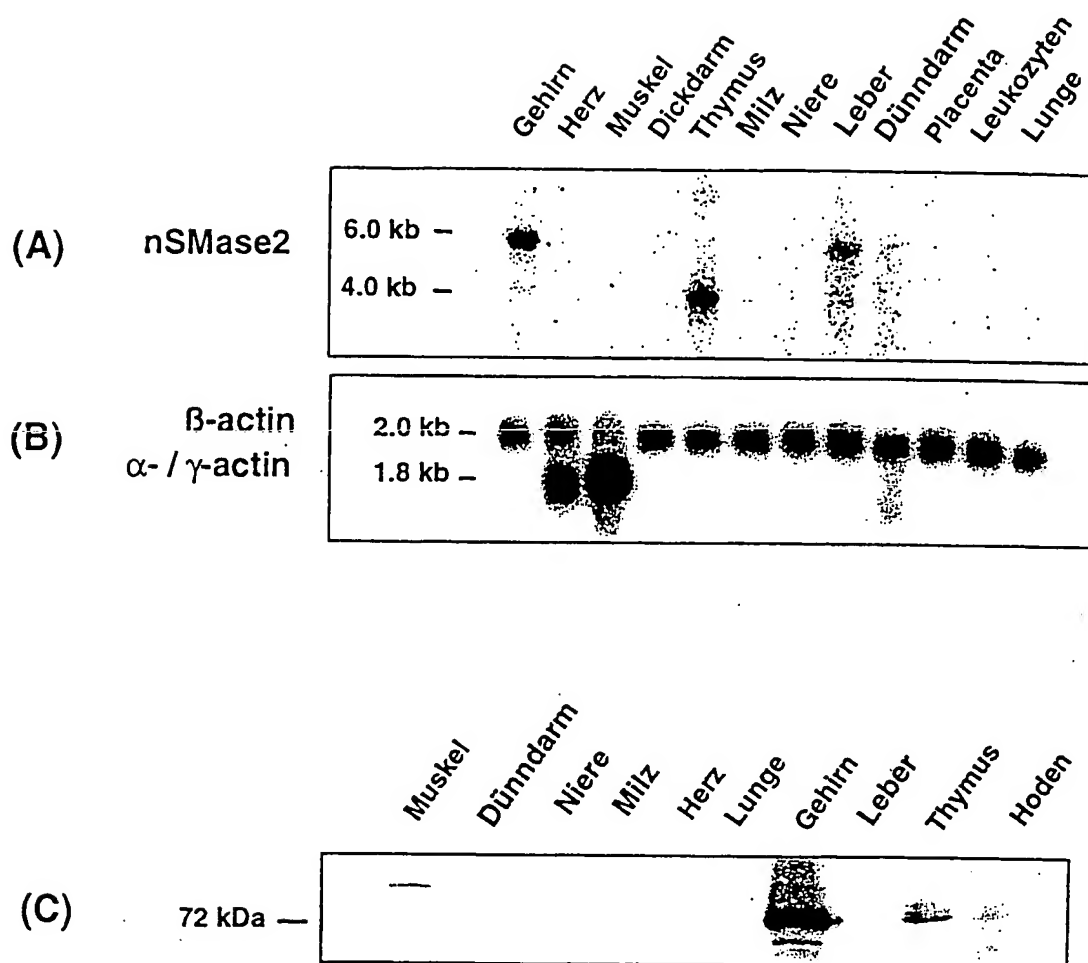


destens einem der Ansprüche 1 bis 7 und/oder einen Antikörper gemäß Anspruch 9 zusammen mit weiteren Hilfsstoffen.

15. Diagnostikmittel enthaltend eukaryontische neutrale Hirn-Sphingomyelinase gemäß Anspruch 8, eine Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 7 und/oder einen Antikörper gemäß Anspruch 9 zusammen mit weiteren Hilfsstoffen.
16. Verwendung der Arzneimittel gemäß Anspruch 14 oder der Diagnostikmittel gemäß Anspruch 15 zur Diagnose und Behandlung von Erkrankungen, die auf einer Über- oder Unterexpression und/oder einer erhöhten oder verminderten Aktivität der eukaryontischen neutralen Hirn-Sphingomyelinase und/oder auf Störungen der Zellproliferation, Zelldifferenzierung und/oder Apoptose beruhen.
17. Verwendung gemäß Anspruch 16, dadurch gekennzeichnet, dass es sich bei den Erkrankungen um Entzündungsprozesse, Zellwachstumstörungen, Krebs und/oder Stoffwechselstörungen wie Störungen der Cholesterinhomöostase (Arteriosklerose) handelt.
18. Verfahren zum Screening von Wirkstoffen dadurch gekennzeichnet, daß die Veränderung der Expression oder Aktivität der eukaryontischen neutralen Hirn-Sphingomyelinase in Zelllinien gemäß Anspruch 10 bei Zugabe von mindestens einer möglichen pharmazeutisch wirksamen Substanz gemessen wird.
19. Verwendung der Zelllinie gemäß Anspruch 10 zur Entwicklung und Prüfung von pharmazeutischen Leitstrukturen.
20. Verfahren zur Herstellung der eukaryontischen neutralen Hirn-Sphingomyelinase gemäß Anspruch 8 durch chemische Peptid-

synthese oder durch Expression in gentechnisch veränderten Organismen, insbesondere in eukaryontischen Expressionssystemen.

21. Verfahren zur Herstellung einer Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 7 durch chemische Synthese oder durch Vervielfältigung in gentechnisch veränderten Organismen.
22. Nukleinsäuren gemäß mindestens einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass es sich um das Gen für eukaryontische neutrale Hirn-Sphingomyelinase handelt und neben codierenden Bereich (Exons) nicht codierende Bereiche (Introns) aufweist.
23. Varianten der eukaryontischen neutralen Hirn-Sphingomyelinase gemäß Anspruch 8.
24. Nukleinsäure gemäß mindestens einem der Ansprüche 1 bis 7 oder 22, dadurch gekennzeichnet, dass es sich um Derivate, Fragmente oder Varianten der Nukleinsäuren handelt.



Figur 1

nSMase2 Maus Protein 655 Reste

MVLYTTPFPNSCLSHAVSWALIFPCYWLVDRLLASFIPTTYEKRQRAD  
DPCCQLFCTVLFTPVYLALLVAALPFAFLGFIFWSPLOSARRPYSYSRL  
EDKNPAGGAALLSEWKGTGAGKSFCFATANVCLLPDSLARLNNVFNTQAR  
AKEIGQRIRNGAARPOIKIYIDSPTNTSISAASFSSLVSPQGGDGSRAVP  
GSIKRTASVEYKGDGGRHPSDEAANGPASGEQADGSLEDSCIVRIGGEEG  
GRPQEADDPAAGSQARNGAGGTPKGQTPNHNQRDGDGSLGSPSASRESL  
VKARAGQDSGGSGEPGANSKLLYKTSVVKKAARRRRHPDEAFDHEVSAF  
FPANLDFLCLQEVFDKRAAAKLKEQLHGYFEYILYDVG VYGCHGCCNFKC  
LNSGLFFASRYPVMDVAYHCYPNGCSFDALASKGALFLKVQVGSTPQDQR  
IVGYIACHTLHAPPEDSAVRCEQLDLLQDWLADFRKSTSSTSTANPEELV  
VFDVICGDLNFDNCSSDDKLEQQHSLFTRYKDPCRLGPGEKPPWAIGTLL  
DTNGLYDEDVCTPDNLQKVLESEEGRREYLAFPTSKSPGAGQKGRKDLLK  
GNRRIDYMLHAEEGLCPDWKAEVEEFSFITQLSGLTDHLPVAMRLMVSA  
GEEEA

Figur 2

nSMase2 Human Protein 655 Reste

MVLYTTPFPNSCLSHCVSWALIFPCYWLVDRLAASFIPPTYEKRQRAD  
DPCCQLQLCTALFTPIYLALLVASLPFAFLGFLFWSPLOQSARRPYIYSRL  
EDKGLAGGAALLSEWKGTGPGKSFCEATANVCLLPDSLARVNNLFNTQAR  
AKEIGQIRINGAARPQIKIYIDSPNTSISAASFSSLVSPQGGDGVARAV  
PGSIKRTASVEYKGDGGRHPGDEAANGPASGDPVDSSSPEDACIVRIGGE  
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AFFPANLDFLCLQEVFDKRAATKLKEQLHGYFEYILYDVG VYGCQGCCSF  
KCLNSGLLFASRYPIMDVAYHCYPNKCND DALASKGALFLKVQVGSTPQD  
QRIVGYIACHTLHAPQEDSAIRCGQLDLLQDWLADFRKSTSSSSAANPEE  
LVAFDVVCGDFNFDNCSSDDKLEQQHSLFTHYRDPCRLGPGEKPPWAIGT  
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GNRRIDYMLHAEGLCPDWKAEVEEFSFITQLSGLTDHLPVAMRLMVSS  
GEEEA

Figur 3

nSMase2 Maus DNA 1968 Reste

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GACCCCTGCTGCCTGCAGCTGTTCTGCACGGTCCTCTTCACGCCAGTCTA  
CCTGGCTCTGCTTGTGGCTGCACTGCCCTTTGCCTTTCTCGGGTTCATCT  
TCTGGTCTCCACTGCAGTCTGCCCGCCGGCCCTACTCCTACTCCCGGCTA  
GAAGACAAGAACCAGCTGGTGGGGCAGCCCTGCTTAGTGAATGGAAGGG  
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GGCAATGGCCGCCGCATCGACTACATGCTGCACGCCGAGGAGGGGCTGTG  
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CCGGCCTGACCGACCACCTCCCCGTGGCCATGCGGCTGATGGTGTCTGCA  
GGGGAAGAGGAGGCGTAG

Figur 4

WO 01/12818

nSMase2 Human DNA 1968 Reste

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GACCCGTGCTGCCTGCAGCTGCTCTGCACTGCCCTCTTCACGCCCATCTA  
CCTGGCCCTCCTGGTGGCCTCGCTGCCCTTTGCGTTTCTCGGCTTTCTCT  
TCTGGTCCCCACTGCAGTCGGCCCGCCGGCCCTACATCTATTACGGCTG  
GAAGAACAAGGGCTGGCCGGTGGGGCAGCCCTGCTCAGTGAATGGAAGGG  
CACGGGGCCTGGCAAAGCTTCTGCTTTGCCACTGCCAACGTCTGCCTCC  
TGCCCGACTCACTCGCCAGGGTCAACAACCTTTTTTAACACCCAAGCGCGG  
GCCAAGGAGATCGGGCAGAGAATCCGCAATGGGGCCGCCCGGCCAGAT  
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TCGACAGCAGCAGCCCGGAGGATGCCTGCATCGTGCGCATCGGTGGCGAG  
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GCCTTCTTCCCGCCAACCTGGACTTCCTGTGCCTGCAGGAGGTGTTTGA  
CAAGCGAGCAGCCACCAATTGAAAGAGCAGCTGCACGGCTACTTCGAGT  
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AAGTGTCTCAACAGCGGCCTCCTCTTTGCCAGCCGCTACCCCATCATGGA  
CGTGGCCTATCACTGTTACCCCAACAAGTGTAACGACGATGCCCTGGCCT  
CTAAGGGAGCTCTGTTTCTCAAGGTGCAGGTGGGAAGCACACCTCAGGAC  
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CCTGCAGAAGGTCCTGGAGAGTGAGGAGGGCCGAGGGAGTACCTGGCGT  
TTCCCACCAGCAAGAGCTCGGGCCAGAAGGGGCGGAAGGAGCTGCTGAAG  
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CCCAGACTGGAAGGCCGAGGTGGAAGAATTCAGTTTTATCACCAGCTGT  
CCGGCCTGACGGACCACCTGCCAGTAGCCATGCGACTGATGGTGTCTTCG  
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Figur 5

## SEQUENZPROTOKOLL

&lt;110&gt; Memorec Stoffel GmbH Medizinisch-Molekulare Entwic

&lt;120&gt; Neutrale Hirn-Sphingomyelinase

&lt;130&gt; Memorec

&lt;140&gt;

&lt;141&gt;

&lt;160&gt; 4

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 655

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 1

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Arg Leu Leu Ala Ser Phe Ile Pro Thr Thr Tyr Glu Lys Arg Gln Arg  
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Pro Val Tyr Leu Ala Leu Leu Val Ala Ala Leu Pro Phe Ala Phe Leu  
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Gly Phe Ile Phe Trp Ser Pro Leu Gln Ser Ala Arg Arg Pro Tyr Ser  
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Tyr Ser Arg Leu Glu Asp Lys Asn Pro Ala Gly Gly Ala Ala Leu Leu  
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Ser Glu Trp Lys Gly Thr Gly Ala Gly Lys Ser Phe Cys Phe Ala Thr  
 115 120 125

Ala Asn Val Cys Leu Leu Pro Asp Ser Leu Ala Arg Leu Asn Asn Val  
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Phe Asn Thr Gln Ala Arg Ala Lys Glu Ile Gly Gln Arg Ile Arg Asn  
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Thr Ser Ile Ser Ala Ala Ser Phe Ser Ser Leu Val Ser Pro Gln Gly  
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Gly Asp Gly Ser Arg Ala Val Pro Gly Ser Ile Lys Arg Thr Ala Ser  
 195 200 205

Val Glu Tyr Lys Gly Asp Gly Gly Arg His Pro Ser Asp Glu Ala Ala  
 210 215 220

Asn Gly Pro Ala Ser Gly Glu Gln Ala Asp Gly Ser Leu Glu Asp Ser  
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Cys Ile Val Arg Ile Gly Gly Glu Glu Gly Gly Arg Pro Gln Glu Ala  
 245 250 255

Asp Asp Pro Ala Ala Gly Ser Gln Ala Arg Asn Gly Ala Gly Gly Thr  
 260 265 270

Pro Lys Gly Gln Thr Pro Asn His Asn Gln Arg Asp Gly Asp Ser Gly  
 275 280 285

Ser Leu Gly Ser Pro Ser Ala Ser Arg Glu Ser Leu Val Lys Ala Arg  
 290 295 300

Ala Gly Gln Asp Ser Gly Gly Ser Gly Glu Pro Gly Ala Asn Ser Lys  
 305 310 315 320

Leu Leu Tyr Lys Thr Ser Val Val Lys Lys Ala Ala Ala Arg Arg Arg  
 325 330 335

Arg His Pro Asp Glu Ala Phe Asp His Glu Val Ser Ala Phe Phe Pro  
 340 345 350

Ala Asn Leu Asp Phe Leu Cys Leu Gln Glu Val Phe Asp Lys Arg Ala  
 355 360 365

Ala Ala Lys Leu Lys Glu Gln Leu His Gly Tyr Phe Glu Tyr Ile Leu  
 370 375 380

Tyr Asp Val Gly Val Tyr Gly Cys His Gly Cys Cys Asn Phe Lys Cys  
 385 390 395 400

Leu Asn Ser Gly Leu Phe Phe Ala Ser Arg Tyr Pro Val Met Asp Val  
 405 410 415

Ala Tyr His Cys Tyr Pro Asn Gly Cys Ser Phe Asp Ala Leu Ala Ser  
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Lys Gly Ala Leu Phe Leu Lys Val Gln Val Gly Ser Thr Pro Gln Asp  
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Gln Arg Ile Val Gly Tyr Ile Ala Cys Thr His Leu His Ala Pro Pro  
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Glu Asp Ser Ala Val Arg Cys Glu Gln Leu Asp Leu Leu Gln Asp Trp  
 465 470 475 480

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 485 490 495

Glu Glu Leu Val Val Phe Asp Val Ile Cys Gly Asp Leu Asn Phe Asp  
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Asn Cys Ser Ser Asp Asp Lys Leu Glu Gln Gln His Ser Leu Phe Thr  
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Arg Tyr Lys Asp Pro Cys Arg Leu Gly Pro Gly Glu Glu Lys Pro Trp  
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Cys Thr Pro Asp Asn Leu Gln Lys Val Leu Glu Ser Glu Glu Gly Arg  
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Lys Gly Arg Lys Asp Leu Leu Lys Gly Asn Gly Arg Arg Ile Asp Tyr  
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Met Leu His Ala Glu Glu Gly Leu Cys Pro Asp Trp Lys Ala Glu Val  
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Arg Leu Ala Ala Ser Phe Ile Pro Thr Thr Tyr Glu Lys Arg Gln Arg  
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Ala Asp Asp Pro Cys Cys Leu Gln Leu Leu Cys Thr Ala Leu Phe Thr  
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Pro Ile Tyr Leu Ala Leu Leu Val Ala Ser Leu Pro Phe Ala Phe Leu  
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Gly Phe Leu Phe Trp Ser Pro Leu Gln Ser Ala Arg Arg Pro Tyr Ile  
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Tyr Ser Arg Leu Glu Asp Lys Gly Leu Ala Gly Gly Ala Ala Leu Leu  
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Ala Asn Val Cys Leu Leu Pro Asp Ser Leu Ala Arg Val Asn Asn Leu  
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 Ser Lys Leu Leu Tyr Lys Ala Ser Val Val Lys Lys Ala Ala Ala Arg  
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 Pro Gln Glu Asp Ser Ala Ile Arg Cys Gly Gln Leu Asp Leu Leu Gln

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&lt;211&gt; 1968

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 3

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&lt;210&gt; 4

&lt;211&gt; 1968

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 4

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ccagtagcca tgcgactgat ggtgtcttcg ggggaggagg aggcatag 1968

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/07889

|  |   |  |
|--|---|--|
| A. CLASSIFICATION OF SUBJECT MATTER  |   |  |
| IPC 7  | C12N15/55<br>A61K38/43  | C12N9/16<br>A01K67/027                             |
| C12N5/10   | C07K16/40   | G01N33/50  |
| According to International Patent Classification (IPC) or to both national classification and IPC  |   |  |
| B. FIELDS SEARCHED   |   |  |
| Minimum documentation searched (classification system followed by classification symbols)  |   |  |
| IPC 7 C12N   |   |  |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  |   |  |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used)   |   |  |
| STRAND, EPO-Internal, WPI Data, PAJ, BIOSIS  |   |  |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT   |   |  |
| Category *   | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.                              |
| Y  | WO 98 37094 A (GENETICS INST)<br>27 August 1998 (1998-08-27)<br>page 7 -page 8<br>page 23, line 7 - line 30<br>claims 20-22<br>Seq Id Nos 7,8               | 1-24   |
| Y  | WO 99 07855 A (HOFMANN KAY ;STOFFEL<br>WILHELM (DE); TOMIUK STEPHAN (DE); MEMOREC<br>ST) 18 February 1999 (1999-02-18)<br>the whole document<br>---<br>-/-- | 1-24   |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.  |   |  |
| * Special categories of cited documents :<br>*A* document defining the general state of the art which is not considered to be of particular relevance<br>*E* earlier document but published on or after the international filing date<br>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>*O* document referring to an oral disclosure, use, exhibition or other means<br>*P* document published prior to the international filing date but later than the priority date claimed<br>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>*&* document member of the same patent family |   |  |
| Date of the actual completion of the international search  |   | Date of mailing of the international search report |
| 19 January 2001  |   | 02/02/2001   |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx: 31 651 epo nl,<br>Fax: (+31-70) 340-3016   |   | Authorized officer<br><br>Ceder, O                 |



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/07889

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| A          | LIU BIN ET AL: "Purification and characterization of a membrane bound neutral pH optimum magnesium-dependent and phosphatidylserine-stimulated sphingomyelinase from rat brain"<br>JOURNAL OF BIOLOGICAL CHEMISTRY, US, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.,,<br>vol. 273, no. 51,<br>18 December 1998 (1998-12-18), pages 34472-34479, XP002140655<br>ISSN: 0021-9258<br>the whole document | 1-24                  |
| A          | HAYASHI ET AL: "ccal is required for formation of growth-arrested confluent monolayer of rat 3Y1 cells"<br>JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD,<br>vol. 272, no. 29, 1997, pages 18082-18086, XP002140656<br>ISSN: 0021-9258<br>abstract; figure 1   | 1-11,<br>20-24        |
| A          | WO 98 28445 A (CHATTERJEE SUBROTO ;UNIV JOHNS HOPKINS (US))<br>2 July 1998 (1998-07-02)<br>abstract; claims 1-30   | 1-24                  |
| A          | ITO M ET AL: "A novel enzyme that cleaves the N-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms"<br>JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD,<br>vol. 270, no. 41,<br>13 October 1995 (1995-10-13), pages 24370-24374, XP002140657<br>ISSN: 0021-9258<br>the whole document                | 1-24                  |
| P, X       | HOFMANN ET AL.: "Cloning and characterization of the mammalian brain-specific, Mg21-dependent neutral sphingomyelinase"<br>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA,<br>vol. 97, no. 11, 23 May 2000 (2000-05-23), pages 5895-5900, XP002157877<br>the whole document   | 1-24                  |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP00/07889

Additional Matter PCT/ISA/210

Continuation of box I.I

Although Claims Nos 16,17 partially relate to a diagnosis method which is carried out on the human or animal body or relate to a method of treatment of the human or animal body, the search was carried out and was based on the cited effects of the compound or composition.

Continuation of box I.1

Rule 39.1. (iv) PCT diagnosis method carried out on the human or animal body

Rule 39.1. (iv) PCT method of treatment of the human or animal body by therapy

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/07889

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)                   | Publication<br>date                    |
|---|---------------------|--|--|
| WO 9837094 A                              | 27-08-1998          | AU 6337398 A<br>EP 0971950 A                 | 09-09-1998<br>19-01-2000               |
| WO 9907855 A                              | 18-02-1999          | AU 9261798 A<br>EP 1021546 A                 | 01-03-1999<br>26-07-2000               |
| WO 9828445 A                              | 02-07-1998          | US 5919687 A<br>AU 5809398 A<br>EP 0948651 A | 06-07-1999<br>17-07-1998<br>13-10-1999 |

# INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PCT/EP 00/07889

## A. KLASIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

IPK 7 C12N15/55 C12N9/16 C12N5/10 C07K16/40 G01N33/50  
A61K38/43 A01K67/027

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

## B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 7 C12N

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

STRAND, EPO-Internal, WPI Data, PAJ, BIOSIS

## C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

| Kategorie* | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile   | Betr. Anspruch Nr. |
|------------|--|--------------------|
| Y          | WO 98 37094 A (GENETICS INST)<br>27. August 1998 (1998-08-27)<br>Seite 7 -Seite 8<br>Seite 23, Zeile 7 - Zeile 30<br>Ansprüche 20-22<br>Seq Id Nos 7,8 | 1-24               |
| Y          | WO 99 07855 A (HOFMANN KAY ;STOFFEL<br>WILHELM (DE); TOMIUK STEPHAN (DE); MEMOREC<br>ST) 18. Februar 1999 (1999-02-18)<br>das ganze Dokument<br>-/-    | 1-24               |

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

☒ Siehe Anhang Patentfamilie

\* Besondere Kategorien von angegebenen Veröffentlichungen :

\*A\* Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

\*E\* älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

\*L\* Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

\*O\* Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

\*P\* Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

\*T\* Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

\*X\* Veröffentlichung von besonderer Bedeutung: die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

\*Y\* Veröffentlichung von besonderer Bedeutung: die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

\*Z\* Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

19. Januar 2001

Absendedatum des internationalen Recherchenberichts

02/02/2001

Name und Postanschrift der Internationalen Recherchenbehörde  
Europäisches Patentamt, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Bevollmächtigter Bediensteter

Ceder, 0

## C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

| Kategorie* | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile   | Betr. Anspruch Nr. |
|------------|--|--------------------|
| A          | <p>LIU BIN ET AL: "Purification and characterization of a membrane bound neutral pH optimum magnesium-dependent and phosphatidylserine-stimulated sphingomyelinase from rat brain"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, US, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.,</p> <p>Bd. 273, Nr. 51,</p> <p>18. Dezember 1998 (1998-12-18), Seiten 34472-34479, XP002140655</p> <p>ISSN: 0021-9258</p> <p>das ganze Dokument</p> <p>---</p> | 1-24               |
| A          | <p>HAYASHI ET AL: "ccal is required for formation of growth-arrested confluent monolayer of rat 3Y1 cells"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD,</p> <p>Bd. 272, Nr. 29, 1997, Seiten 18082-18086, XP002140656</p> <p>ISSN: 0021-9258</p> <p>Zusammenfassung; Abbildung 1</p> <p>---</p>   | 1-11,<br>20-24     |
| A          | <p>WO 98 28445 A (CHATTERJEE SUBROTO ;UNIV JOHNS HOPKINS (US))</p> <p>2. Juli 1998 (1998-07-02)</p> <p>Zusammenfassung; Ansprüche 1-30</p> <p>---</p>  | 1-24               |
| A          | <p>ITO M ET AL: "A novel enzyme that cleaves the N-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD,</p> <p>Bd. 270, Nr. 41,</p> <p>13. Oktober 1995 (1995-10-13), Seiten 24370-24374, XP002140657</p> <p>ISSN: 0021-9258</p> <p>das ganze Dokument</p> <p>---</p>               | 1-24               |
| P,X        | <p>HOFMANN ET AL.: "Cloning and characterization of the mammalian brain-specific, Mg<sup>2+</sup>-dependent neutral sphingomyelinase"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA,</p> <p>Bd. 97, Nr. 11, 23. Mai 2000 (2000-05-23), Seiten 5895-5900, XP002157877</p> <p>das ganze Dokument</p> <p>-----</p>   | 1-24               |

# INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen PCT/EP 00 07889

WEITERE ANGABEN

PCT/ISA/ 210

Fortsetzung von Feld I.1

Obwohl die Ansprüche 16,17, teilweise, sich auf ein Diagnostizierverfahren, das am menschlichen/tierischen Körper vorgenommen wird, und/oder sich auf ein Verfahren zur Behandlung des menschlichen/tierischen Körpers, beziehen, wurde die Recherche durchgeführt und gründete sich auf die angeführten Wirkungen der Verbindung/Zusammensetzung.

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Fortsetzung von Feld I.1

Regel 39.1(iv) PCT - Diagnostizierverfahren, die am menschlichen oder tierischen Körper vorgenommen werden.

Regel 39.1(iv) PCT - Verfahren zur therapeutischen Behandlung des menschlichen oder tierischen Körpers

# INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internationales Aktenzeichen

PCT/EP 00/07889

| Im Recherchenbericht<br>angeführtes Patentdokument |   | Datum der<br>Veröffentlichung | Mitglied(er) der<br>Patentfamilie |           | Datum der<br>Veröffentlichung |
|--|---|-------------------------------|-----------------------------------|-----------|-------------------------------|
| WO 9837094   | A | 27-08-1998                    | AU                                | 6337398 A | 09-09-1998                    |
|  |   |                               | EP                                | 0971950 A | 19-01-2000                    |
| WO 9907855   | A | 18-02-1999                    | AU                                | 9261798 A | 01-03-1999                    |
|  |   |                               | EP                                | 1021546 A | 26-07-2000                    |
| WO 9828445   | A | 02-07-1998                    | US                                | 5919687 A | 06-07-1999                    |
|  |   |                               | AU                                | 5809398 A | 17-07-1998                    |
|  |   |                               | EP                                | 0948651 A | 13-10-1999                    |

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